UNIVERSIDAD AUTÓNOMA DE MADRID
DEPARTAMENTO DE BIOQUÍMICA

Extending the life-span of mice with dysfunctional telomeres

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1. ACKNOWLEDGEMENTS

First of all I would like to thank my supervisor Maria Blasco for giving me the great opportunity to do my PhD in her unique laboratory, for believing in my work and giving me such a learning experience that I will benefit from now and in the future.

I am very grateful to Peter Klatt for his endless support – *deine Unterstützung war unschätzbar!*

I would also like to thank Manolo for his scientific discussions, Rosa and Ester for caring about the mice, Puri for her contribution to the G&D paper, Nacho for teaching me the stem cell techniques, Nani F. and Pedro for their fundamental cooperation with the mouse experiments, Diego for his patience with the microscope, Susa for making life much easier and other people from the CNIO for their support and help.

Thanks very much to Luis, Carolyn, Águeda, Katerina, Martina and Han for their generous contributions to the writing of the thesis.

Then my lab-friends Águeda, Isabel, Alejo, Christoph, Toñi, and later arrivals, Ana, Carolyn, Han, Ralph and all the people of the "telomeres and telomerase" and the "tumor suppressor" groups, Pablo, Daniela, Paula, Maribel, Cristina, Manuel C., Martina, Katerina, Dani, Arancha Antonio, Nacho, Rosa, Ester, Fabian, Raquel, Elisa, Susana Llanos, Susana Velasco, Elsa, Gerdine, Stefan, Nani M., Alejandro, from now and the past, Rebecca, Andrés, Susana Gonzalo, Silvia, Sonia, Constanza, Marta, Maria-Luisa, Caye, Roberta, Marta B., Ander, Elena, Isabel G., Susana Gonzalez - thank you very much for all your help and friendship!

And, my "life-long" friends, I do not need to mention your names (many of you are mentioned above!) and put simply, many thanks that I always can count on you! I am very grateful to my friends "en rojo sobre ruedas" and from ACA who helped keep my life in balance.

A huge thanks also to my family for always supporting my dreams and ideas and standing by me. I dedicate this thesis to my parents.

2. PREFACE

Telomeres are protective structures that form the ends of chromosomes. They are nucleoprotein complexes that consist of tandem repeats of the sequence TTAGGG (in vertebrates), bound to a specific set of diverse proteins [1, 2]. Telomere repeats are generated by telomerase, an enzyme composed of a protein subunit, the telomerase reverse transcriptase (Tert), and a RNA subunit (Terc). The reverse transcriptase elongates the telomere using the RNA molecule as a template [3]. Telomeres and telomerase are important determinants of stem cell behavior and, therefore, are important factors in cancer and aging. Stem cells proliferate out of their niche, the cellular micro-environment that supplies the needs of the stem cells, and repopulate the tissue as differentiated cells. As a consequence of aging and concomitant telomere shortening the mobilization of stem cells out of their niche decreases, leading to reduced tissue regeneration which finally results in organ failure. Several diseases, including dyskeratosis congenita (DKC), are caused by a deficiency in telomerase in stem cells [4-10]. Reconstitution of telomerase has been proposed as a potential gene therapy to prevent or reverse the impact of these types of diseases. We have tested whether telomerase re-introduction into late generation telomerase-deficient mice would prevent telomere-associated stem cell proliferation defects. In these mice stem cell mobilization and proliferation defects were rescued, which was accompanied by normal tissue regeneration, normal body weight, normal life span, and normal telomere length homeostasis. Importantly, telomerase re-introduction was sufficient to warrant a normal life span of these mice without increased cancer incidence.

A multiprotein complex known as shelterin shapes and protects the telomere end to prevent the double-stranded structure from being recognized as DNA damage. Shelterin components include several proteins involved in DNA repair and the DNA damage response (DDR) [11]. Mismatch repair (MMR) proteins are involved in the DNA repair of base-base mismatches and insertion/deletion loops (IDLs), and have been shown to interact with the protein WRN which plays a significant role in telomere maintenance [12]. Mismatch repair deficiency enhances telomerase-independent survival in yeast and telomerase inhibition in a MMR-deficient human cancer cell line caused telomerase-independent telomere elongation (alternative telomere lengthening, ALT) [13, 14]. A role for MMR proteins in signaling DNA damage through the p53 pathway has also been proposed [15-17]. Here we describe the function of the MMR protein PMS2 in the cellular response to short and dysfunctional telomeres. Mice doubly-deficient for PMS2 and telomerase show an increased life span coinciding with a decrease in the severity of degenerative pathologies in the intestine, compared to mice lacking telomerase alone. This finding correlated with higher cellular proliferation levels and attenuated p21 induction in the small intestine. For the first time, these results support the concept that the mismatch repair protein PMS2 acts in the same pathway as p21, mediating cell-cycle arrest and aging caused by telomere shortening.

3. RESUMEN

Los telómeros son unas estructuras protectoras que conforman los extremos de los cromosomas. Son unos complejos nucleoproteicos consistentes en una serie de repeticiones en tándem de la secuencia TTAGGG (en los vertebrados) a las que se une un conjunto específico de diversas proteínas [1, 2]. Las repeticiones teloméricas son generadas por la telomerasa, una enzima compuesta por una subunidad proteica, la transcriptasa reversa (Tert), y una subunidad de ARN (Terc), que sirve como molde a la transcriptasa reversa para alargar los telómeros [3]. Tanto los telómeros como la telomerasa son determinantes importantes en el comportamiento de las células madre y, por ende también lo son en los procesos de cáncer y envejecimiento. Las células madre, para poder repoblar los tejidos en forma ya de células diferenciadas, tienen que abandonar su nicho, el micro-entorno que les suministra todos los aportes que necesitan. El envejecimiento y el acortamiento telomérico asociado ocasionan una disminución en la movilización de las células madre hacia el exterior de sus nichos, lo que resulta en una capacidad de regeneración tisular disminuida que culmina en un fallo órgánico. La deficiencia en telomerasa de las células madres es la causa de varias enfermedades humanas, como, por ejemplo, la disqueratosis congénita (DKC) [4-10]. Se ha propuesto que la reconstitución de la telomerasa como potencial terapia génica para prevenir o revertir el impacto de este tipo de enfermedades. Para probarlo, ensayamos la reintroducción de la telomerasa en generaciones tardías de ratones deficientes en esta enzima y logramos la recuperación de las capacidades de movilización y proliferación de las células madre, acompañadas también por una regeneración tisular, un peso corporal y una homeostasis de la longitud telomérica normal. Es importante resaltar que también se alcanzó una expectativa de vida normal sin incremento de la tumorigénesis.

Para prevenir que los telomeros sean reconocidos como una ruptura de la doble cadena del ADN existe un complejo multiproteico conocido como *shelterin* que protege y da forma a los extremos teloméricos. Algunas de las proteínas que intervienen en la respuesta del daño del DNA y su reparación, como las proteínas de corrección de los apareamientos erróneos (MMR), interaccionan con la proteína WRN la cual desempeña una función significativa en el mantenimiento de los telómeros [12]. Se ha propuesto para las proteínas MMR una funcionalidad en la señalización del daño en el ADN a través de la ruta de p53 [15-17]. Su deficiencia conduce a un aumento de la supervivencia independiente de telomerasa en levaduras y, por otro lado, en células tumorales humanas, carentes de telomerasa, resulta en una elongación de los telómeros por ALT [13, 14]. En este trabajo de tesis describimos la función de la proteína PMS2 (de MMR) en la respuesta celular frente a telómeros cortos y no funcionales. Ratones deficientes en telomerasa y PMS2 exhibieron una extensión de su vida media acompañada por un descenso en la severidad de las patologías degenerativas de intestino, con respecto a los ratones deficientes sólo en telomerasa. Estos resultados correlacionan con un mayor nivel de proliferación celular y una atenuación en la inducción de la proteína p21 en el intestino delgado. Este trabajo establece por primera vez que la proteína PMS2 de MMR actúa en la misma ruta

que p21, mediando la parada en el ciclo celular y el envejecimiento originado por el acortamiento de los telómeros y abre nuevos campos de investigación en estos dos procesos fundamentales íntimamente relacionados que son el cáncer y el envejecimiento.

4. CONTENTS

5. ABBREVIATIONS

ALT	alternative lengthening of the telomeres
APBs	ALT-associated PML bodies
ATM	ataxia telangiectesia mutated
ATR	ATM and Rad3 related
ATRIP	ATR interacting protein
BRCA1	breast cancer 1
Cdc25	cell-division cycle 25
Cdkn1a	Cyclin-Dependent Kinase Inhibitor 1a
Chk1	checkpoint homolog 1
Chk2	checkpoint homolog 2
CO-FISH	chromosome orientation - fluorescence in situ hybridization
D-loop	displacement loop
DDR	DNA damage response
DNA DSB	DNA double-strand break
DKC1	dyskerin protein
DKC	dyskeratosis congenita
DMBA	7, 12-dimethylbenz(a)anthracene
ES	embryonic stem cells
EXO1	exonuclease-1
GHKL	gyrase/Hsp90/histidine-kinase/MutL
GI-tract	gastro-intestinal tract
HF	hair follicle
HFSCs	hair follicle stem cells
HMGB1	high mobility group box 1
HP1 γ	heterochromatin protein 1
HR	homologous recombination
HU	hydroxyurea
IDL	insertion/deletion loop
IFE	inter-follicular epidermis
IF proteins	intermediate filament proteins
IR	ionizing radiation
K5	keratin 5
K14	keratin 13
Ki67	Kiel 67

LRC	label-retaining cells
MDC1	mediator of DNA damage checkpoint 1
MEFs	mouse embryonic fibroblasts
MLH	mutL homolog
MMR	mismatch repair
MSH	MutS homolog
MSI	microsatellite instability
NBS1	nijmegen breakage syndrome 1
NHEJ	non-homologous end-joining
PARP-2	poly(ADP-ribose) polymerase
PCNA	proliferating cell nuclear antigen
PINX1	Pin2/TRF1 interacting protein X-1
PML	promyelocytic leukemia protein
PMS	post-meiotic segregation protein
Pol δ	polymerase δ
POT-1	protection of telomerase 1
PTOP/PIP1	POT1 and TIN2 organizing protein
Q-FISH	quantitative fluorescence in situ hybridization
RAP1	repressor/activator protein 1
RFC	replication factor C
RPA	replication factor A
SKY	spectral karyotyping
ssDNA	single-strand DNA
T-loop	telomere loop
TA cells	transit amplifying cells
TANK1	tankyrase 1
TANK2	tankyrase 2
TIN2	TRF1-interacting nuclear factor
TopBP1	topoisomerase binding protein 1
TPA	12-O-tetradecanoylphorbol 13-acetate
TRF	terminal restriction fragment
TRF1	telomeric repeat binding factor 1
TRF2	telomeric repeat binding factor 2
Trp53	transformation related protein 53
T-SCE	telomeric-sister chromatide exchange
Terc	telomerase RNA component (mouse)
Tert	telomerase reverse transcriptase (mouse)

UV- light	ultraviolet-light
XPF	xeroderma pigmentosum, complementation group F
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1
53BP1	p53 Binding Protein 1
γ-H2AX	γ-H2A histone family, member X

6. INTRODUCTION

6.1. Telomeres – Background

Telomeres are nucleoprotein structures located at the ends of chromosomes to protect chromosomal DNA from repair, recombination, and degradation activities [1, 3]. In vertebrates, telomeres are made up of tandem repeats of the TTAGGG sequence bound to a series of specific proteins (see chapter 3.3.2.) [1].

6.1.1. Structure

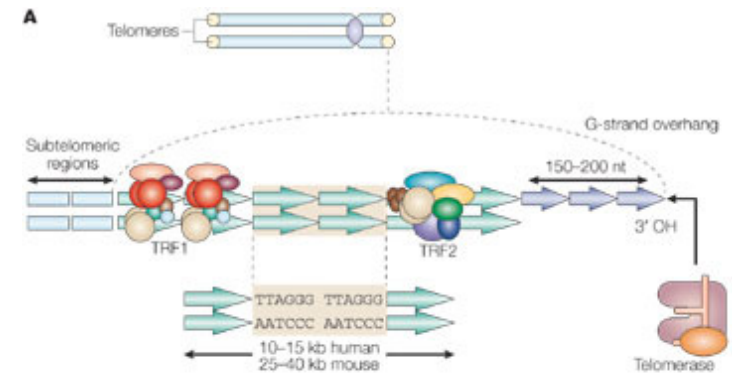

Figure 1: The structure of mammalian telomeres (closer description in the text) [18]

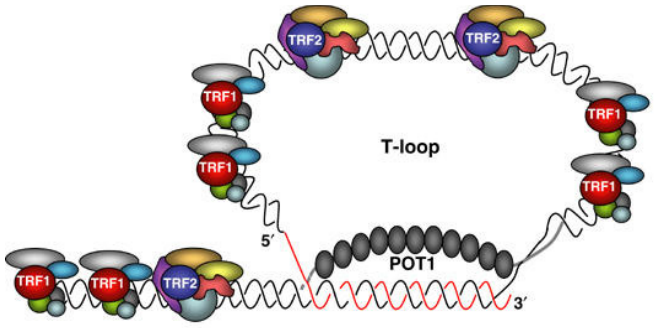

Figure 2: Scheme showing the telomere in a T-loop conformation together with protein complexes at the mammalian [19].

Telomeres do not encode genes and are situated nearby the gene-poor subtelomeric regions (Fig. 1). The length of the telomeres varies from species to species. While in humans they are around 10-15 kb in length, in mice they reach up to 25-40 kb on average. The TRF1 and TRF2 proteins (telomere repeat binding factors 1 and 2, respectively) can directly bind to the TTAGGG repeats and can build complexes with other factors (described in more detail in chapter 3.3.2.) (reviewed in [18]). Telomeres end in a highly-structured G-rich single strand of about 150 to 200 nucleotides known as the **G-strand overhang** [20]. One model that has been proposed to account for the unique structure of telomere ends is the T-loop model. It relays in a loop been built by the annealing of the single-stranded G-strand with the double-stranded region of the telomere (Fig. 2). This structure may function to protect the chromosome ends, and might also serve to restrict the access of telomerase [21-23]. Loss of either TTAGGG repeats or telomere-binding factors results in impaired

chromosome end protection. The consequences are chromosomal end-to end fusions and loss of cell viability [24, 25].

Telomeres continuously shorten due to the loss of TTAGGG repeats during replication of telomeric DNA. Leading strand DNA synthesis results in blunt ends and incomplete replication occurs at the 5' end of the lagging strand during DNA synthesis. The consequence of this differential replication of the leading and lagging strands is the formation of a 3' overhang concomitant to a loss of telomeric DNA. This telomere shortening with each cell division cycle is called the **"end-replication problem".** Critically short telomeres are then recognized by the DNA-damage repair machinery as DNA lesions and the cell either dies or enters senescence. In those cells where telomerase is active, telomere shortening to a critical length is prevented [26-28].

6.1.2. Telomere maintenance mechanisms

6.1.2.1. Maintenance by telomerase

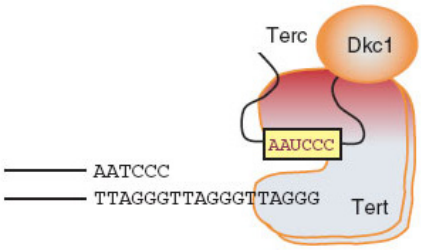

Figure 3: The components of the telomerase enzyme are shown (extended description in the text) [29].

Telomerase is a reverse transcriptase and can elongate the telomere by using an RNA molecule as template [3]. The enzyme consists of a protein subunit, the telomerase reverse transcriptase (Tert), and a RNA subunit (Terc) (Fig. 3). The telomerase complex is stabilized by the dyskerin protein (DKC1). Mutations in this protein, or in Tert or Terc, cause the disease dyskeratosis congenita [30, 31].

Telomere elongation by telomerase proceeds with the recognition of the the 3´-OH group at the end of the G-strand overhang by telomerase, followed by *de novo* DNA synthesis using the RNA molecule, Terc, as template [2, 31]. Telomerase can thus elongate the 3' end solving the problem of progressive loss of telomeric DNA that would have arisen due to cell division. Additionally, due to its capacity to polymerise single-strand DNA, telomerase can also synthesize the G-strand. Unicellular organisms have unlimited amounts of telomerase, in contrast to most multicellular eukaryotes which are endowed with only limited amounts of this enzyme [32]. This restricted availability of telomerase is considered to be responsible for the observed telomere shortening concomitant to cell division during tissue regeneration [18, 31]. Investigation of a telomerase-deficient mouse model showed that short telomeres resulted in stem cell defects [33], impaired tissue regeneration and decreased tumorigenesis [18], while telomerase overexpression has exactly the opposite effects [34-36].

6.1.2.2. Alternative lengthening of telomeres (ALT)

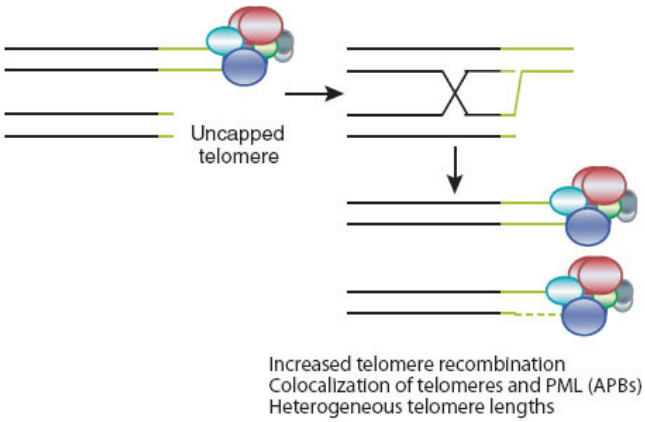

Figure 4: Alternative telomere lengthening by homologous recombination [29].

Yeast and mammals can maintain telomere length by a mechanism known as ALT which involves recombination-mediated DNA replication and is independent of telomerase (Fig. 4). Two independent ALT pathways exist in yeast that can be distinguished by the involvement of either the Rad50 or the Rad51 group of genes [37] and that differ in the resulting length of the telomere [38]. A characteristic of ALT-positive cells in humans is a heterogeneous telomere length distribution consisting of unusually short and long telomeres. Also, the promyelocytic leukemia protein (PML), the ALT-associated PML bodies (APBs) co-localize at telomeres in these cells [39, 40]. APBs are subnuclear structures consisting of the PML associated with linear and circular forms of telomeric DNA, certain telomere-binding proteins and additional proteins that have a role in DNA synthesis and recombination [41]. POT1, TRF2 [18, 36, 42, 43] and many proteins of the homologous recombination DNA-repair pathway have a role in ALT [37-40, 44-46]. ALT could be observed in cultured mouse embryonic fibroblasts (MEFs) [47, 48], embryonic stem (ES) cells of telomerase-deficient mice [33] and in B-cell development. Thus, ALT is utilized in both primary and immortalized cell lines [49]. Interestingly, ALT cannot improve the life span of telomerase-deficient mice although it could in telomerase-deficient yeast cells [45]. This suggests that in multicellular organisms ALT mechanisms alone are not enough to prevent telomeric dysfunction.

6.2. Telomeres and Telomerase function in Cancer and Aging

6.2.1. Mouse models

Telomerase-deficient mice were generated by deletion of the murine *Terc* gene, encoding the RNA component of the telomerase [24, 50]. This deletion abrogates telomerase activity in the affected mice. Importantly, the heterozygous or the first homozygous generation of $Terc^{-/-}$ mice does not differ from wildtype mice concerning their phenotype. However, after several generations the amount of critically short telomeres increases [24, 25, 47, 49, 51-54], giving rise to chromosomal fusions and either cell growth arrest or apoptosis. Tissues with high replicative rates are often affected. These include germ cells (infertility), the gastrointestinal tract (sever mucosal atrophy), skin (alopecia, grey hair), the immune system (splenic atrophy and lack of germinal centre formation after immunization), or the

haematopoietic precursors (growth disadvantage in competitive repopulation assays) [25]. This often results in a segmental progeroid syndrome and decreased viability (50 % of late generation $Terc^{-/-}$ mice in the BL6 background die by 5 months of age). Importantly, late generation telomerase-deficient mice exhibit a resistance when they are treated following a multi-stage skin carcinogenesis protocol using 7,12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol 13-acetate (TPA) [55]. The telomerase-deficient mouse model provides an excellent model for studying the implication of telomerase and telomeres in tumorigenesis and aging.

Telomerase is thought to be responsible for the establishment of "normal" telomere length homeostasis in stem cell compartments of adult tissues [31, 34, 35, 56, 57]. A high number of stem cells were found in the epidermis and hair follicle of late generation telomerase-deficient mice, whereas the number of stem cells was lower in telomerase-proficient mice. This finding could be explained by mobilization defects of the stem cells related to telomerase deficiency. Consequences of telomerase-deficiency were decreased wound-healing, hair graying and a lower cancer incidence related to defective tissue regeneration [25, 51, 52, 55]. Based on the knowledge gained from mouse models, telomere shortening within the aging process in human tissues affects tissue homeostasis by impairing the mobilization of adult stem cells. Conversely, overexpression of telomerase in Tert transgenic mice caused enhanced stem cell mobilization [34]. This leads to an augmented cell proliferation, increased skin thickness and a higher colony formation potential of keratinocytes resulting in an increased tissue fitness.

6.2.2. Stem cells

Stem cells have the ability to divide and renew themselves over the lifetime of an organ, they are unspecialized and can give rise to specialized cell types. Stem cells can be grouped into embryonic stem cells and adult stem cells. Embryonic stem cells are pluripotent, which means that they can become all cell types of the body and even have the potential to generate an entire organism. Adult stem cells are multipotent, they are undifferentiated and located in tissues where they are responsible for the long-term maintenance and regeneration of the tissue.

6.2.2.1. Stem cell niche

A stem cell niche is defined as the cellular microenvironment that fulfills all the requirements for stem cell maintenance. Importantly, the niche offers a protection against several *stimuli*, that may lead to differentiation or apoptosis. Niches have to coordinate stem cell quiescence and activity and regulate stem cell production to avoid uncontrolled proliferation which can lead to cancer formation. [58]. Progenitor or transit amplifying (TA) cells have to be continuously produced by stem cells. These TA cells have the ability to differentiate and to regenerate the corresponding tissue. Certain *stimuli*, such as wounding, can induce the differentiation of these cells. Importantly, the stem cell pool is

continuously refilled although expansion is avoided. This mechanism protects the organism against tumor formation [59]. Well characterized stem cell niches include the intestinal stem cell niche, the hematopoietic stem cell niche and the hair follicle epidermal stem cell niche.

6.2.3. Telomeres, stem cells, aging and cancer

Cellular damage contributes to **cancer and aging** and so, protective mechanisms against damage provide a safeguard against both cancer and aging [60]. Mechanisms that protect against cancer include limiting the proliferative potential of cells, but can lead to enhanced aging.

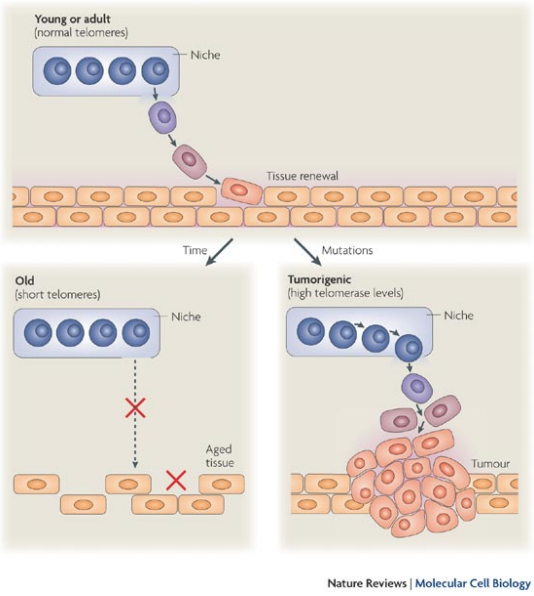

Figure 5: A model for the implication of telomeres and telomerase in stem cell biology (broader description provided in the text). Blue circles are stem cells, orange squares represent differentiated cells [60].

Figure 5 depicts a model explaining stem cell mobilization and tissue regeneration in cancer and aging. Young or adult organisms with a normal telomere length possess functioning stem cells and can consequently regenerate tissue. It is hypothesized that in older organisms (bottom left) the proliferative potential is affected due to critical shortening of the telomeres of the stem cells. Subsequently, stem cells no longer mobilize out of their niches and the tissue cannot be efficiently regenerated, resulting in organ failure. A positive effect of low stem cell mobilization would be the minimization of cancer formation caused by high proliferation (bottom right). Organisms that express a high level of telomerase enjoy an enhanced stem cell mobilization potential, higher tissue regeneration and therefore higher fitness. The potentially higher lifespan is, however, negatively affected by the higher risk to develop cancer [60]. Multiple organ defects are characteristic of telomere-driven aging and a consequence of stem cell failure resulting in tissue regeneration defects [18].

6.2.4. Telomeres and stem cell defects

A number of human diseases are known to be associated with the lack of telomerase in stem cells. A defect of the telomerase RNA template gene causes dyskeratosis congenita (DKC) and results in premature telomere shortening. Bone marrow failure, intestinal disorder, or malignancies before turning 50 years of age are typical symptoms in these patients. By positionial cloning of the gene DKC1, which encodes the protein dyskerin, the former was found to be defective in persons with X-

linked DKC. Dyskerin forms a complex with telomerase and presumably affects telomerase activity [5]. In a family with autosomal-dominant DKC a 3´deletion in the TERC gene was identified to be responsible for the illness. DKC is characterized by the occurrence of aplastic anemia, a disorder of the bone marrow that, in this disease, contains mainly fat and only very few haematopoietic cells. Patients suffering aplastic anemia present germ-line mutations in Terc and Tert [8, 61]. Dyskeratosis congenita patients are highly susceptible to developing cancer, occurring in 10 percent of the cases [62]. Additionally, genomic instability can cause tumor development [63, 64]. Interestingly, tissues with a high turnover rate are especially prone to tumor formation. In summary, a consequence of telomere shortening is stem cell failure which can be prominently observed in tissues with a high turnover, including skin and bone marrow.

6.2.5. Can restoration of telomerase activity cure stem cell defects?

A promising approach for the treatment of age-related diseases caused by critical telomere shortening may be the re-introduction of telomerase activity. This idea was already examined using the telomerase-deficient mouse model. Late generation telomerase-deficient mice, G3 *Terc*$^{-/-}$, with short telomeres and severe proliferative defects were crossed with *Terc*$^{+/-}$ mice. This resulted in late generation telomerase-deficient offspring, G4 *Terc*$^{-/-}$, that disposed over one set of chromosomes with normal telomeres from the *Terc*$^{+/-}$ parent. The other set of chromosomes remained with critically short telomeres from the G3 *Terc*$^{-/-}$ parent. The chromosomes of littermates, reconstituted with one allele of the terc gene, *Terc*$^{+/-}$, all had detectable telomeres [65]. In mice reconstituted with telomerase activity the critically short telomeres were elongated, the chromosomal instability rescued and severe proliferative defects were prevented [65]. However, the effects of telomerase re-introduction on stem cell behavior have not yet been fully investigated. Organ homeostasis and organismal survival are consequences of the capacity of stem cells to regenerate the corresponding tissue. The goal of this project was to study if the restoration of telomerase activity would be enough to ameliorate stem cell defects. Telomerase was therefore re-introduced into late generation telomerase-deficient mice and the biology of epidermal stem cells, a well established model for the exploration of stem cell biology, was investigated.

6.2.6. Adult stem cells in the skin and the hair follicle

Two stem cell populations within the hair follicle and in interfollicular regions have been identified that regenerate the epidermal layer and its associated tissues. The skin layers are regenerated by stem cells residing in the basal layer of the skin. The hair follicle stem cells (HFSC) are located in the hair bulge and are responsible for hair and sebaceous gland renewal [66].

6.2.6.1. The skin

Figure 6 shows the dermal and epidermal components of mammalian skin. Proliferation takes place in the basal layer of the epidermis. The cells gradually differentiate and migrate until they reach the top layer, known as the *stratum corneum*. Cells from the basal layer enter the spinous layer, where they lose their mitotic activity but in turn, they contribute to hardening and solidifying of the epithelium. Keratins produced by the cells support this process of strengthening. The cells move towards the skin surface and after terminal differentiation, the cells undergo a "programmed cell death" similar to normal apoptosis [67]. The *stratum corneum* contains the dead flattened squames which are continuously replaced by inner cells differentiating outwards. Thereby an effective barrier to harming influences from outside and a protection of fluids and tissues inside the organism is assured. The differentiation from the basal layer to squames requires 10-14 days in mouse skin [68]. Initially, it was thought that stem cells are present in the whole basal layer, but after closer examination only about 10-12 percent of the cells were identified as putative stem cells [69].

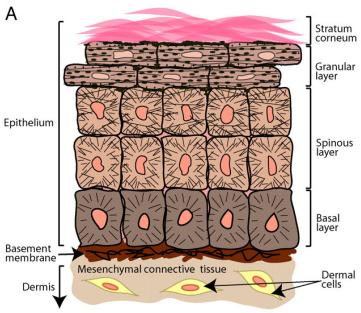

Figure 6: The skin epithelial histology The different layers of the epithelium are shown (extended description is provided in the text) [58, 66].

6.2.6.2. The hair follicle

Figure 7 shows the structure of the hair follicle which is enclosed by dermal cells. Multipotent stem cells are located in the bulge below the sebaceous gland. These cells are thought to produce lineages of the hair follicle, sebaceous gland, and the epidermis. The hair follicle matrix is situated around the dermal papilla and consists of transient amplifying cells that differentiate to build up the inner root sheath and the hair shaft [66]. Within the hair cycle HFSC continuously approach to the dermal papillae to form the growing hair [70]. The hair cycle consists of cycles of growth (anagen), regression (catagen) and rest (telogen). In the anagen phase an entire hair is constructed, in catagen phase apoptosis takes place and proliferation decreases, and in telogen phase neither apoptosis nor proliferation is detectable [71].

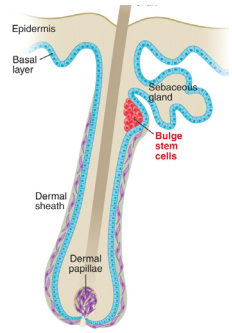

Figura 7: The hair follicle structure [58].

6.3. Molecular mechanism of Telomere and Telomerase function in Cancer and Aging

6.3.1. DNA damage signaling due to short telomeres

Telomeres represent DNA double-strand breaks (DSBs) with an additional single-strand DNA (ssDNA) end. In mammals, several DNA damage proteins are found at the chromosome ends. These proteins are not responsible for DNA repair, cell cycle arrest, or apoptosis that usually occurs in response to damaged DNA but instead, these proteins are incorporated into a nucleoprotein complex called shelterin that protects and stabilizes the telomere [11].

As mentioned in chapter 6.1.1, telomeres shorten with every cell division as a consequence of the end-replication-problem. Uncompleted DNA replication, nuclease cutting at the chromosome ends, and the lack of telomerase all affect telomere length [72]. When telomeres cannot fulfill their protective function, as in the case of critical telomere shortening, the cells enter senescence reflecting a DNA damage checkpoint response [73]. Long telomeres are protected by shelterin complexes which are composed of 3 subunits, TRF1, TRF2, and POT1, and these subunits are connected to each other by TIN2, TPP1, and Rap1 [1]. Short telomeres have lost this protective complex and they are recognized as DNA damage by the DNA damage machinery. Consequently, important proteins which mediate the eukaryotic DNA-damage response, such as ataxia telangiectasia mutated (ATM) and Atm and Rad3-releated (ATR) kinases are activated [73]. It was demonstrated that TRF2 prevents ATM mediated DNA damage response, whereas POT1 avoids activation of ATR [74].

6.3.1.1. The “cancer-aging hypothesis”

The “cancer-aging hypothesis” accounts for the effect of the mechanisms that prevent aging but conversely, increase the level of tumorigenesis. V*ice versa*, in aged tissues the rates of tumor growth would be lower.

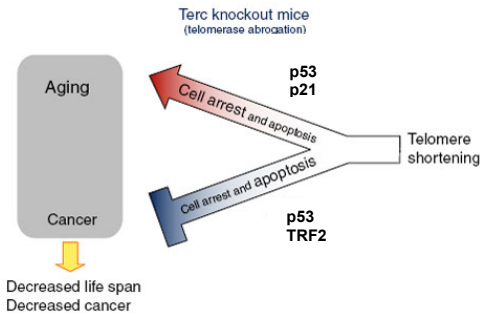

Figure 8: Telomere shortening and p53, p21, and TRF2 (detailed explanation in the text) (modified from [29]).

Mice deficient for telomerase and tumor suppressor proteins are important tools to study cancer and aging. It has been previously shown that telomerase-deficient mice with a defect in a tumor suppressor protein mostly developed decreased tumorigenesis due to the tumor suppressive effect of short telomeres that provokes cell cycle arrest and apoptosis [75-82]. Importantly, p53 mutant and TRF2 over-expressing mice with short telomeres have to be excluded, because in these mice the cancer incidence is further increased (Fig. 8) [42, 83, 84]. Degenerative pathologies caused by telomerase-deficiency are improved in a p53-deficient background as well as in

the absence of p21 [78, 83, 84]. These differential results of p53 and p21 are explained by the observation that p21 abrogation causes a recuperation of proliferation but not of apoptosis in mice with critically short telomeres [29].

Dysfunctional telomeres, DNA damage, the activation of oncogenes, and reactive oxygen species (ROS) induce the p53 pathway and result in apoptosis or senescence, which are two recognized tumor suppressor mechanisms that are also involved in stress response [85]. p21, the inhibitor of cyclin-dependent kinases, is a downstream target of p53 and leads to cell-cycle arrest. Interestingly, p21 does not seem to be responsible for the tumor suppressor properties of p53, although this protein appears to be very important in the cellular response to DNA damage and other stresses [78]. A persistent stress signal would have a pro-aging effect on stem-cells due to p21 function, whereas only a transient stress signal would provoke stem cell repair and therefore have an anti-aging effect (Fig. 9). A promising approach would be to separate the age-promoting and tumor-suppressive properties of p53 with the help of p21 [86]. As a consequence of p21 inhibition, the aging process would be stopped or at least delayed, while tumor suppressive activities of p53 would stay intact [85].

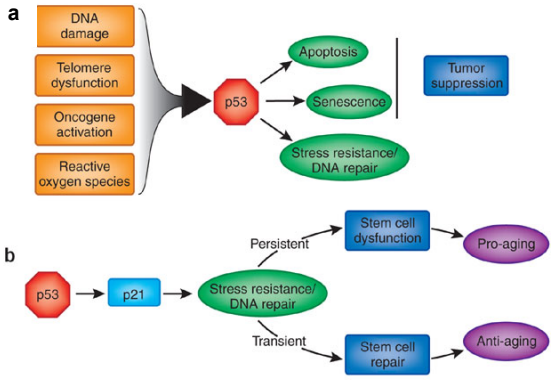

Figure 9: The role of p21 in p53 response (a) p53 responds to several *stimuli* and provokes the induction of senescence, apoptosis or a DNA damage or stress response. Apoptosis and senescence have been linked to tumor suppression, whereas the role of the stress response in preventing cancer is not clearly established. (b) p21 is an effector of p53 and seems to induce a reversible cell-cycle arrest, allowing DNA repair or stress resistance. This arrest can either protect or compromise stem cell function and aging depending on the persistence of a stimulus activating p53 [85].

6.3.2. Telomeres – proteins

Proteins associated with the telomere can be classified into those that bind directly to the double-stranded TTAGGG region, and others that bind to the single-stranded G-strand or bind indirectly. TRF1 and TRF2 [87] belong to the former group, while proteins like TIN2 [88], TANK1 and 2 [89-91], PINX1 [92], PTOP/PIP1 [93], the Mre11 complex [94], the DNA-PK complex [95], PARP2 [96] and belong to the latter group.

6.3.2.1. TRF1 regulates telomere length

The protein TRF1 (Telomeric repeat binding factor 1) is involved in the control of telomere length. It forms a complex with several other proteins including TIN2 (TRF1-interacting nuclear factor) [88], TANK1 and TANK2 (tankyrase 1 and 2) poly(ADP)-ribosylases [90, 97], POT-1 (protection of telomerase 1), PTOP/PIP1 (POT1 and TIN2 organizing protein), and RAP1 (repressor/activator protein 1). TRF1 and TRF2 are connected indirectly through TIN2 [18] (Fig. 10). Interestingly, a targeted deletion of TRF1 does not provoke changes in telomere-length nor in telomere protection. However, the deletion of this protein is embryonic lethal [98]. An explanation for these phenotypes is that TRF1-interacting proteins compensate for TRF1 dysfunction. TANK1 is one of these TRF1-interacting proteins and, additionally, is involved in cell division. Knockdown of TANK1 causes separation of sister chromatids at centromeres and arms but the sister chromatids remain associated with the telomeres provoking mitotic arrest [99]. ADP-ribosylation of TRF1 by TANK1 and TANK2 diminished its ability to bind to telomeric DNA *in vitro* [90]. Inhibition of TRF1 by TANK1 is controlled by TIN2. Partial knockdown of TIN2 resulted in telomere elongation [100] whereas mice with an inactivated TIN2 gene die at the embryonic stage [101]. TIN2 domains associate with HP 1 γ, supporting the hypothesis that TIN2 domains may promote chromatin compaction [102]. TIN2 interacts with TRF1 and TRF2, suggesting that the functions of both proteins are linked by TIN2 [100, 103, 104]. TRF1 interacts with the single-stranded telomeric DNA-binding protein POT1 thus transmitting information about telomere length to the telomere terminus, the single-stranded 3´-overhang, to regulate telomerase [105]. POT1 is recruited to the telomere by forming a heterodimer with PTOP [93, 106, 107]. PINX1 (Pin2/TRF1 interacting protein X-1) is another TRF1-interacting protein that inhibits telomerase activity and affects tumorigenicity, therefore functioning as a putative tumor suppressor [108]. Additionally, there are some human tumor types where the expression of TRF1, TRF2, TIN2, POT1 and TANK1 is altered [109-113].

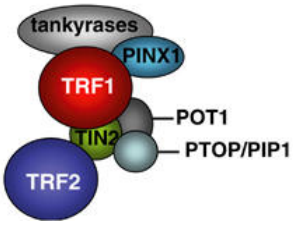

Figure 10: The components of the telomere repeat binding factor 1 (TRF1) complex [18].

6.3.2.2. TRF2 protects and regulates the telomere

TRF2 is involved in telomere protection and telomere-length regulation (Fig.11) [18]. TRF2 has a key role protecting the chromosome ends from end-to-end fusion and avoiding the loss of the G-strand overhang [114]. Increased amounts of TRF2 have been found in several tumors [109, 113]. Over-expression of TRF2 results in a phenotype in mouse skin similar to *xeroderma pigmentosum* in humans. These mice exhibit a severe reaction when exposed to day light [115]. Keratinocytes over-expressing TRF2 present increased chromosomal instability associated with very short telomeres and the loss of the G-strand overhang.

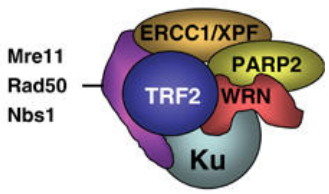

Figure 11: The components of the telomere repeat binding factor 2 (TRF2) [18].

Furthermore, telomere loss in TRF2-overexpressing mice is mediated by XPF/ERCC1, a nuclease implicated in repair of UV-induced DNA damage by nucleotide excision repair [115]. XPF/ERCC1 cuts and removes the telomere 3´-overhang upon inhibition of TRF2 [116]. End-to-end fusions, provoked by non-homologous end-joining, are typical aberrations related to dysfunctional telomeres [117, 118]. Another type of chromosomal aberration, translocations are the consequence of binding of dysfunctional telomeres to a double-stranded break [119]. Interestingly, TRF2 has a role in establishing proper telomere structure indicating that TRF2 can convert model telomeres into t-loop structures [120]. hRAP is recruited to the telomere by TRF2 and negatively regulates telomere length [121, 122].

Interestingly, several of the proteins recruited by TRF2 are related to DNA repair processes. For example, PARP-2, an important player in base excision repair and other repair pathways, physically binds to TRF2 and functions as a negative regulator of TRF2 [123]. Through co-immunoprecipitation studies an interaction between human Ku70 and TRF2 has been demonstrated [124]. Immortalized human cell lines lacking telomerase activity display ALT-associated PML bodies (APBs) that contain, in addition to other proteins, TRF1, TRF2, and WRN [125]. APBs also contain replication factor A (RPA), RAD51, and RAD52 which all have a role in DNA synthesis and recombination [41]. Moreover, the MRE11 complex, consisting of RAD50, MRE11, and NBS1, is important for homologous recombination (HR), non-homologous end-joining pathways (NHEJ) and DNA double-strand break (DSB) repair, and also interacts with TRF2 [94].

TRF2 is required to prevent telomeres from activating DNA damage checkpoints. This protein can specifically bind to ATM and alter the ATM-dependent DNA damage response. Therefore it was proposed that TRF2 can inhibit ATM activation at telomeres [126]. Inhibition of TRF2 or telomere shortening results in association of the telomeres with ATM and other DNA damage response factors like p53-binding protein1 (53BP1) and phosphorylation of γ-H2AX [73, 127, 128].

6.3.3. DNA damage – DNA damage pathways and signaling

The preservation of an intact genome is fundamental for the life of an organism. Even the smallest damage provokes a signaling cascade that results in DNA repair [129]. DNA damage can trigger DNA repair, DNA damage checkpoints, transcriptional response, and apoptosis [130].

DNA repair genes can be subgrouped into genes associated with signaling and regulation of DNA repair and genes associated with distinct repair mechanisms. DNA repair pathways can be further classified into excision repair pathways and double-strand break repair pathways.

Excision repair pathways can be further divided into **nucleotide excision repair**, **base excision repair** (single-strand break repair is often considered a part of base excision repair), and **mismatch repair** (described in more detail in section 3.3.3.2.). In general, these repair pathways operate via recognition of the damage, excision of the lesion by incision of the DNA phosphodiester backbone, re-synthesis of the missing section by DNA polymerase (with the use of the complementary DNA strand as template), and in a final event the strand is closed by ligation [131].

Double-stranded breaks are caused by numerous damaging agents, such as chemicals and ionizing radiation. This form of damage is very harmful to the organism. **Double-strand break repair pathways** can be divided in **non-homologous end joining (NHEJ)** and **homologous recombination (HR)**. NHEJ is responsible for the ligation of two broken DNA ends with no or minimal homology. This repair system is error-prone due to the absence of an intact DNA template. In contrast, in HR a homologous sequence serves as a template making this repair machinery error-free [131].

6.3.3.1. DNA damage checkpoints

Cell cycle checkpoints ensure the fidelity of cell division in eukaryotic cells. Checkpoints specifically refer to halting the cell cycle to allow thus for time to repair and prevent replication of damaged DNA, thereby avoiding cancer formation. DNA damage checkpoints are signal transduction pathways activated by DNA damage and are responsible for changes in cell cycling, regulation of DNA repair genes, inducing transcription, and when cells cannot cope with the damage, the triggering of apoptotic cell death pathways [132].

Broadly, DNA damage checkpoints can be grouped into three categories: **sensors, signal transducers, and effectors** (Fig. 12). It is important to note that there is no clear cut separation because certain proteins can overtake functions of several components.

Two phosphoinositide 3-kinase-like (PI3k-like) checkpoint kinases have been identified as important DNA damage **sensors**. For each of the DNA-damage-responsive checkpoints either ATM (ataxia telangiectesia mutated) or ATR (ATM and Rad3 related) is necessary [133].

ATM is important in the response to ionizing radiation (IR) and is thought to be activated mainly in response to double-strand breaks. ATM phosphorylates many proteins like Chk2, p53 [134, 135], NBS1 [136], BRCA1 [137] and itself [138]. ATR, on the other hand, is activated by UV light, DNA methylating agents and replication inhibitors like hydroxyurea (HU) and aphidicolin [139]. Repair of DNA through this pathway commonly results in extended regions of ssDNA. ATR phosphorylates nearly all of the same proteins which are phosphorylated by ATM and functions by forming a complex with ATRIP (ATR Interacting Protein) Additionally, it was described that ATRIP senses DNA damage and mediates ATR activation through recognition of RPA-ssDNA complexes [140].

Another DNA-damage sensor is the 9-1-1 (Rad9-Rad1-Hus1) complex, a ring-like structured homotrimer that shares sequence similarity with PCNA, the DNA polymerase processivity factor. PCNA surrounds the DNA and binds DNA polymerase δ to increase its processivity. Due to the structural similarities it was suggested that the 9-1-1 complex has the same function as PCNA [141]. Rad17, on the other hand, is similar to the large subunit of RFC (replication factor C), a PCNA accessory factor [142]. This observation indicates a role for Rad17-RFC in the loading of the 9-1-1 complex onto damaged DNA [133, 143].

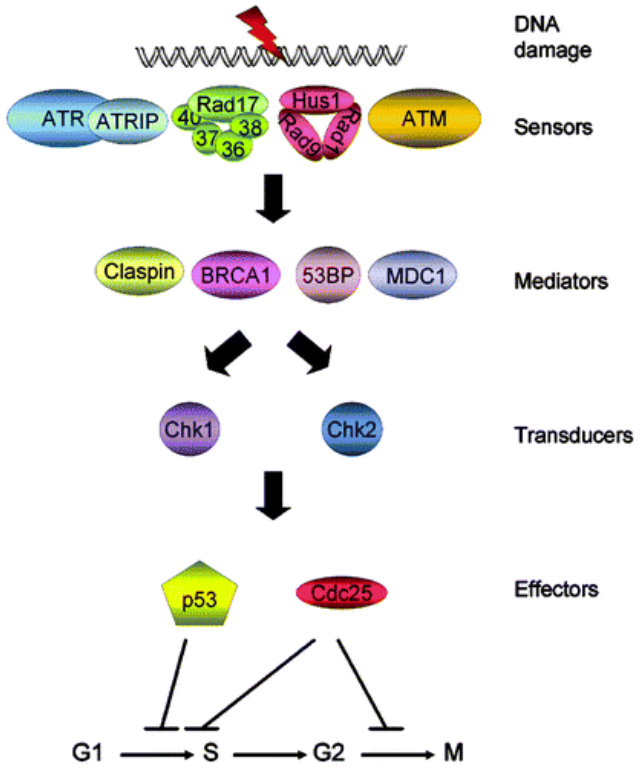

Figure 12: Components of the DNA damage checkpoints in human cells. The damage is detected by sensors, mediators transduce the signal to transducers and effectors directly participate in certain checkpoints of the cell cycle [129].

The function of the **mediators** is to associate with damage sensors and signal transducers. ATM interacts with mediator proteins like the p53 binding protein 53BP1 [144, 145], the topoisomerase binding protein, TopBP1 [146], and the mediator of DNA damage checkpoint 1, MDC1[147-149].

In humans there are the two **signal transducers**, the kinases Chk1 and Chk2 which can inhibit cell cycle progression [133, 150, 151]. In mammalian cells, Chk2 is phosphorylated in an ATM-dependent manner provoked by IR [152, 153]. Conversely, Chk1 is phosphorylated in response to ATR [139, 154]. However, the functions of both proteins are overlapping.

Effector proteins include the phosphotyrosine phosphatase Cdc25 and p53. These proteins enable checkpoint activation by direct regulation of important cell cycle proteins at both the G1 to S phase and G2 to M phase transitions.

6.3.3.2. Mismatch repair (MMR)

MMR is an important DNA repair pathway that fulfills several tasks:

- MMR corrects base-base mismatches and insertion/deletion loops during DNA synthesis
- MMR is involved in the cellular response to several types of DNA damage
- MMR proteins are involved in mitotic and meiotic recombination
- MMR proteins play a role in triplet repeat instability
- MMR proteins have a function in the generation of immunoglobulin diversity [155-157]

Defects in the mismatch repair pathway are the reason for the development of typical and atypical hereditary nonpolyposis colon cancer. Additionally, defects in this repair machinery are responsible for the occurrence of 15-25 % of sporadic tumors [158, 159].

6.3.3.2.1. Mechanism of mismatch repair in eukaryotes

The MMR machinery recognizes base-base mismatches and insertion/deletion loops (IDLs) and corrects them in an error-free way. The sequence comprising the error of the newly synthesized strand is degraded and the DNA polymerase can create a new copy using the remaining strand as a template.

Microsatellite instability is the consequence of mismatch repair defects. Characteristically, microsatellites consist of repeated-sequence motifs, like $[A]_n$ or $[CA]_n$. These motifs are distributed over the genome and are the reason for the formation of heteroduplex DNA molecules, produced by the annealing of complementary single strands derived from different parental duplex molecules during DNA synthesis. A microsatellite sequence can provoke the dissociation of primer and template strand to generate incorrect reannealing. The microsatellite-repeat units in the template and in the newly synthesized strand differ and cause the formation of an **insertion/deletion loop** (IDLs).

Additionally, errors of the DNA polymerases that escape proofreading, are responsible for the appearance of **base-base mismatches** [157]**.**

The mechanism (Fig. 13):

Three proteins are responsible for the initiation of the repair process in human cells. MSH2 and MSH6 form the heterodimer **MutSα** and recognize base-base mismatches and IDLs of one or two extrahelical nucleotides. **MutSβ** consists of MSH2 and MSH3 and binds to larger IDLs [155, 160-162]. Like other proteins involved in DNA repair, the MSH proteins are ATPases with a Walker ATP-binding motif [163, 164].

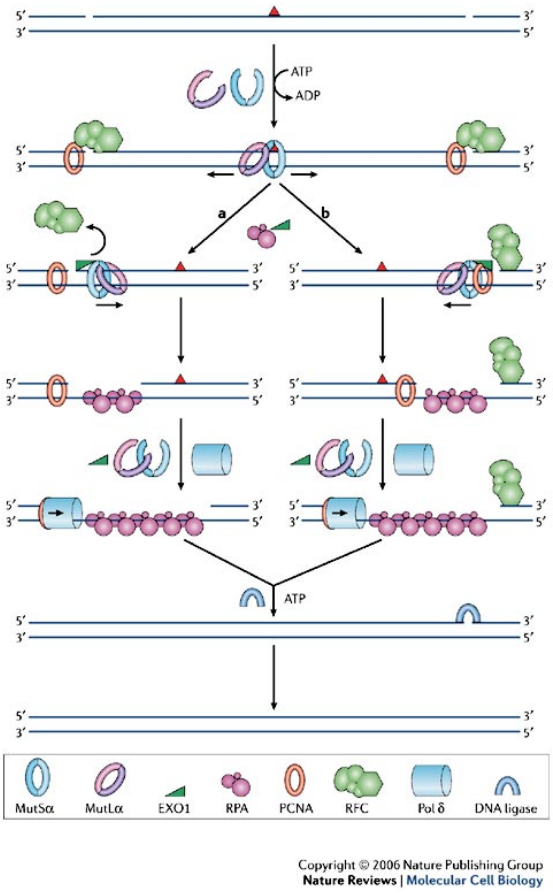

Figure 13: The human mismatch-repair system. Proteins involved in mismatch repair (MMR) are Mut Sα or Mutsβ, MutLα, replication protein A (RPA), exonuclease-1 (EXO1), proliferating cell nuclear antigen (PCNA), replication factor C (RFC), DNA polymerase δ (Pol δ), and DNA ligase I (see text for details) [157].

Four **MutL** homologues are found in human cells, MLH1, MLH3, PMS1 (post-meiotic segregation protein-1) and PMS2. These proteins are ATPases of the gyrase/Hsp90/histidine-kinase/MutL (GHKL) family [165] and form three different heterodimers. The complex MLH1-PMS2 (MutLα) is the most important one. Until recently, the exact mechanism of mismatch repair was unclear [157].

Numerous biochemical and structural studies have led to the development of the following **model** [157] (Fig. 13) in which MutSα or Mutsβ recruits MutLα and the complex starts diffusing in upstream and downstream directions away from the lesion.

Upstream of the lesion, replication factor C (RFC) is probably replaced after exonuclease-1 (EXO1) is loaded and EXO1 degrades the strand in the 5′→ 3′direction. Replication protein A (RPA) is responsible for the stabilization of the single-stranded gap. MutSα, on one hand, stimulates EXO1 activity whilst MutLα inhibits EXO1 after the mismatch is eliminated. DNA polymerase δ (Pol δ) together with proliferating cell nuclear antigen (PCNA) fills the gap and DNA ligase I connects the remaining ends and thereby finishes the process.

Downstream of the lesion, it is thought that the complex, MutSα, MutLα, EXO1, PCNA and RFC (and possibly high mobility group box 1 (HMGB1)) are responsible for the degradation of the error-

containing strand. The single-stranded DNA would be protected by RPA and DNA polymerase δ could fill the gap. Again, DNA ligase I finishes up by connecting the remaining ends [157].

6.3.3.2.2. MMR in DNA-damage signaling (Figure 14)

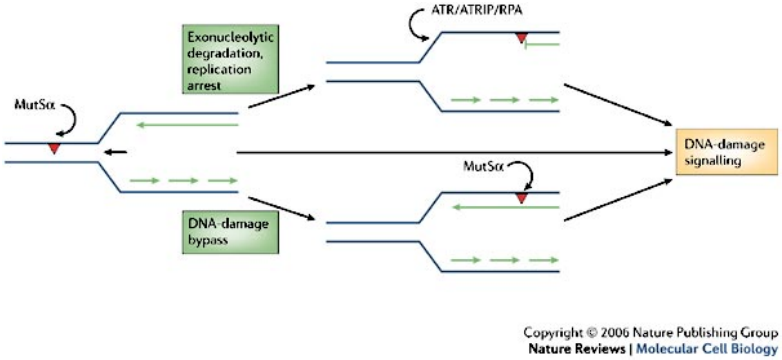

Figure 14: MMR in DNA-damage signalling: In this model mismatch-repair proteins (MutSα) can recognize DNA damage (red triangle), signal to the cell-cycle checkpoint machinery directly and induce cell-cycle arrest and apoptosis (middle). Damage cannot be processed and the replication-fork arrests, single-stranded DNA regions recruit replication protein A (RPA) and ATR-interacting protein (ATRIP), activate the ATR kinase and checkpoint kinase CHK1 (top). Or damage could be bypassed by an error-prone DNA polymerase and secondary lesions would cause DNA damage signalling (bottom).

There are several lesions that provoke a DNA damage response as a result of mismatch repair processing. These include S_N1 DNA methylators, 6-thioguanine, 5-fluoro-deoxyuridine, cisplatin, ultraviolet light, and several carcinogens. MMR processing of these lesions is ineffective, leading to multiple rounds of futile repair, cell cycle arrest, and ultimately apoptosis [156].

6.3.3.2.3. MMR and recombination

Homologous recombination (HR) involves the pairing of single strands of DNA derived from different parental duplexes, giving rise to a heteroduplex DNA molecule. If the sequences of the two strands are not identical, the heteroduplex will contain mismatches. The consequences of DNA mispairing is genomic instability via chromosomal translocations, deletions, or inversions [166, 167].

The mechanisms of HR (Figure 15)

HR starts with the detection of a DSB and the processing of the DNA by an unknown exonuclease on both ends gives rise to 3' single-strand DNA. Replication protein A (RPA) avoids the formation of secondary structures by binding and protecting the single-strand DNA. Rad51, with the help of Rad52, Rad55 and Rad57, takes over from RPA and the invasion of the homologous double-strand commences. In an ATP-dependent process, the ssDNA strands swap partners. Rad54 has an important but not very well known role. Probably, it is responsible for opening the chromatin structure to permit strand invasion and might enable the release of Rad51 from the double-strand DNA. A D-loop

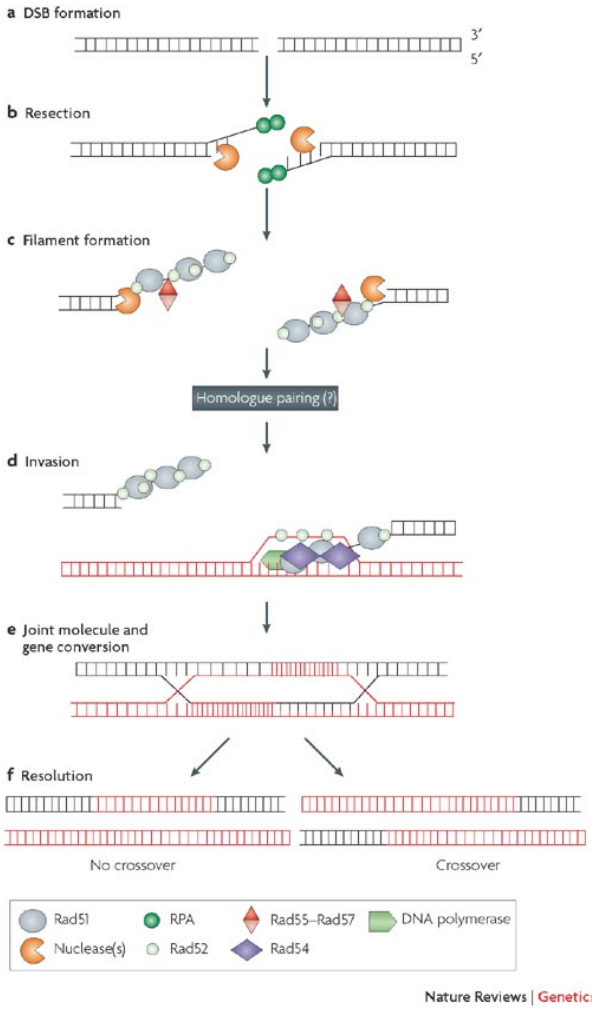

Figure 15: Homologous recombination (HR) in DSB-repair. The figure depicts the mechanism including the involved proteins (detailed description is provided in the text) [168].

(displacement loop), containing heteroduplex DNA, is built and DNA synthesis begins primed by the 3' single-strand DNA ends. HR finishes the process by cutting the double Holliday junction intermediate and final ligation [168].

The mismatch repair machinery is involved in several processes related to homologous recombination. It can correct mismatches, abort the recombination event, and remove non-homologous single-strand tails [169].

Gene conversion occurs due to **MMR** and the proofreading activity of DNA polymerase at the heteroduplex of recombination intermediates. In this process the sequence of one chromosome is replaced by a homologous sequence from another chromosome [169].

The eukaryotic MMR system has an **anti-recombination activity** that blocks recombination between diverged sequences in mitosis and meiosis [170]. Studies in yeast demonstrated that a single mismatch is enough to inhibit recombination between otherwise identical sequences in a mismatch repair-dependent manner. Additional mismatches affect the recombination rate in a cumulative negative manner until a level of divergence is reached where the mismatch repair machinery is no longer necessary to inhibit recombination [171].

Another task of the MMR machinery is the **removal of non-homologous tails** generated in homologous recombination. A single-stranded tail with a 3' end enters a homologous duplex DNA molecule and serves as primer for DNA synthesis. However, if the 3' end is not homologous to the invaded duplex, the non-homologous segment has to be removed before DNA synthesis starts [169]. Although MMR proteins do not play a direct role it was shown that MSH2 and MSH3 cooperate with the RAD1-RAD10 endonuclease to remove nonhomologous 3' tails in yeast [172, 173].

7. INTRODUCCIÒN

7.1. Los telómeros – Antecedentes

Los telómeros son unas estructuras nucleoproteicas localizadas en los extremos de los cromosomas para proteger al ADN cromosomal de las actividades de reparación, recombinación y degradación [1, 3]. En los vertebrados los telómeros están formados por un número de repeticiones en tándem de la secuencia TTAGGG, a las que se han unido una serie de proteínas específicas (ver capítulo 3.3.2) [1].

7.1.1. Estructura

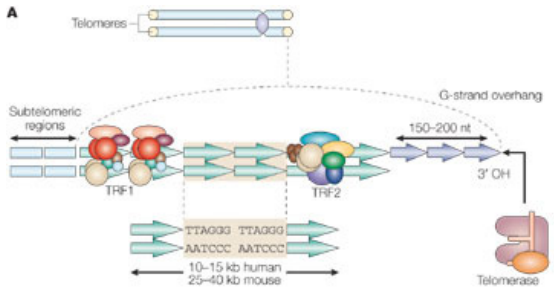

Figura 1: La estructura de los telómeros de mamíferos (descripción detallada en el texto) [18].

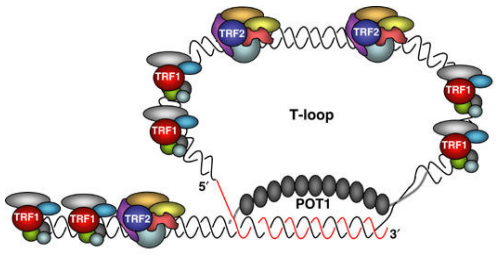

Figura 2: Ilustración esquemática del telómero de mamíferos en la conformación de *T-loop* junto con los complejos proteicos del telómero [19].

Los telómeros no contienen genes codificantes y están situados en las proximidades de las regiones subtelómericas con bajo contenido en genes (Fig. 1). La longitud de las telómeros varía con las especies, mientras que en humanos su longitud aproximada es de unas 10-15 kb, en ratones llegan a alcanzar de promedio las 25-40 kb. Las proteínas TRF1 y TRF2 (factores de unión a telómeros 1 y 2, respectivamente) se unen directamente a las repeticiones TTAGGG y establecen complejos con otros factores (se describe en mayor detalle en el capítulo 3.3.2) (revisado en [18]). Los telómeros finalizan en una hebra de cadena simple muy estructurada de unas 150 a 200 bases de longitud y con alto contenido en bases G por lo cual se conoce como la **hebra-G protuberante** [20]. La estructura de los extremos de los telómeros es singular y para explicarla se han propuesto varios modelos, uno de ellos es el modelo del *T-loop*. El mismo se basa en la generación de un *loop* surgido tras el anillamiento de la hebra-G protuberante (monocatenaria) con la región de cadena doble del telómero (Fig. 2). La función de esta estructura podría ser la de proteger los extremos de los cromosomas, y también podría servir para restringir el acceso de la telomerasa [21-23]. La pérdida de las repeticiones TTAGGG, o bien de los factores de unión a los telómeros resulta en una protección defectuosa de los extremos de los cromosomas. Como consecuencia, ocurren fusiones cromosómicas entre extremos, conduciendo a una pérdida de la viabilidad celular [24, 25].

Durante la replicación del ADN telomérico se produce una pérdida de las repeticiones TTAGGG y los telómeros, por tanto, se acortan continuamente. Por un lado, la síntesis del ADN de la cadena adelantada ocasiona extremos romos; por otro lado, durante la síntesis del de la cadena retrasada la replicación es incompleta en el extremo 5'. La consecuencia de la replicación diferencial de las cadenas adelantada y retrasada es la formación de una protuberancia en el extremo 3' concomitante a la pérdida del ADN telomérico. Este acortamiento telomérico con cada ciclo de división celular se conoce como el "**problema de la replicación de los extremos**". La maquinaria de reparación del ADN interpreta que los telómeros críticamente cortos son un tipo de lesión en el ADN y la célula o muere o senesce. En aquellas células en las que la telomerasa está activa se evita que los telómeros se acorten hasta alcanzar una longitud crítica [26-28].

7.1.2. Mecanismos para el mantenimiento de los telómeros

7.1.2.1. Mantenimiento mediante la telomerasa

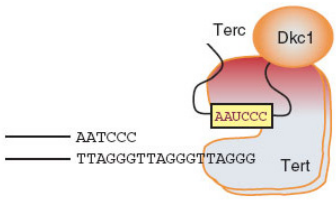

Figura 3: Componentes del enzima telomerasa (descripción más extensa en el texto) [29].

La telomerasa es una transcriptasa reversa que emplea moléculas de ARN como moldes para alargar los telómeros [3]. El enzima consiste en una subunidad proteica, la transcriptasa reversa de la telomerasa (Tert), y una subunidad de ARN (Terc) (Fig. 3). La proteína disquerina (DKC1) dota de estabilidad al complejo de la telomerasa. Las mutaciones en DKC1, en Tert o en Terc, ocasionan la enfermedad denominada disqueratosis congénita [30, 31].

La telomerasa comienza el alargamiento de los telómeros con el reconocimiento del grupo 3'-OH del el extremo de la hebra-G protuberante, y prosigue con la síntesis de novo del ADN para la que emplea de molde a la molécula de ARN, Terc [2, 31]. De esta forma la telomerasa es capaz de alargar el extremo 3' y de resolver el problema de la pérdida progresiva de ADN que, si no, hubiese surgido como consecuencia de la división celular. Puesto que la telomerasa posee la capacidad para polimerizar ADN monocatenario, sintetiza adicionalmente la hebra-G. Los organismos unicelulares tienen cantidades ilimitadas de telomerasa, al contrario que la mayoría de los eucariotas multicelulares, cuya dotación de este enzima es limitada [32]. Se considera que la disponibilidad restringida de telomerasa es la responsable del acortamiento telomérico concomitante a la división celular que se observa durante la regeneración tisular [18, 31]. La caracterización de un modelo murino deficiente en telomerasa puso de manifiesto que los telómeros cortos ocasionaban defectos en las células madre

[33], una regeneración tisular impedida y una tumorigénesis disminuida [18], mientras que la sobre-expresión de la telomerasa producía justamente los efectos opuestos [34-36].

7.1.2.2. Alargamiento alternativo de los telómeros (ALT)

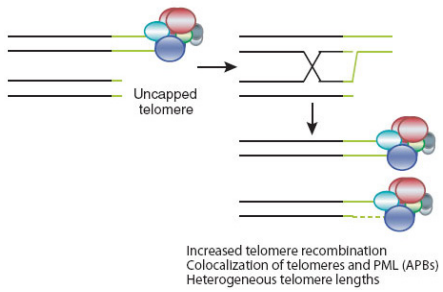

Figura 4: Alargamiento alternativo de los telómeros mediante recombinación homóloga [29].

En las levaduras y los mamíferos se puede mantener la longitud telomérica mediante un mecanismo conocido como ALT *(alternative lengthening of the telomere)* que involucra una replicación del ADN mediada por recombinación y que es independiente de la telomerasa (Fig. 4). En las levaduras existen dos rutas de ALT independientes que se distinguen por la participación del grupo de genes de Rad50 o del grupo de Rad51 [37] y que difieren en la longitud telomérica resultante [38]. Una característica de las células humanas positivas para ALT es una distribución heterogénea de la longitud telomérica que abarca telómeros inusualmente cortos y largos. En estas células se observa también una colocalización en los telómeros de la proteína promielocítica de la leucemia (PML) y de los cuerpos de PML asociados a ALT (APBs) [39, 40]. Los APBs son unas estructuras subnucleares que contienen PML asociado a formas lineales o circulares de ADN telomérico, ciertas proteínas de unión a los telómeros y unas proteínas adicionales implicadas en la síntesis y la recombinación del ADN [41]. En ALT participan también las proteínas POT1 y TRF2 [18, 36, 42, 43] y muchas proteínas de la ruta de reparación del ADN mediante recombinación homóloga [37-40, 44-46]. ALT ha sido detectado en cultivos de fibroblastos embrionarios de ratón (MEFs) [47, 48], en las células madre embrionarias de ratones deficientes en telomerasa [33] y en el desarrollo de los linfocitos B. ALT, por lo tanto, se utiliza tanto en líneas celulares primarias como en las inmortalizadas [49]. Es interesante notar que, aunque en las células de levaduras deficientes en telomerasa ALT puede prolongar su período de vida, no puede hacerlo en los ratones carentes de telomerasa [45]. Esto sugiere que, en los organismos multicelulares, ALT por sí solo no es suficiente para prevenir la no funcionalidad telomérica.

7.2. Función de los telómeros y la telomerasa en el cáncer y el envejecimiento

7.2.1. Modelos murinos

Para estudiar la función de los telómeros y la telomerasa en los procesos de cáncer y envejecimiento se generaron ratones deficientes en telomerasa mediante la deleción del gen *Terc* murino, que codifica al componente ARN de la telomerasa [24, 50]. Esta deleción elimina la actividad telomerasa en los ratones afectados. Es de relevancia que los ratones heterocigóticos o la primera generación

homocigótica de los ratones Terc$^{-/-}$ no difieren de los ratones silvestres en lo que se refiere a sus fenotipos. Tras varias generaciones, sin embargo, aumenta el número de telómeros críticamente cortos [24, 25, 47, 49, 51-54], lo que ocasiona fusiones cromosómicas y bien una parada en el crecimiento celular bien apoptosis. Los tejidos de elevada capacidad proliferativa están a menudo afectados. Entre éstos se cuentan las células germinales (infertilidad), el tracto gastrointestinal (atrofia severa de la mucosa), piel (alopecia, cabellos grises), el sistema inmune (atrofia del bazo y perdida de la formación de centros germinales tras la inmunización), o los precursores hematopoyéticos (desventaja de crecimiento en los ensayos de competencia de repoblación) [25]. Lo anterior resulta a menudo en el síndrome de progeria segmentada y una viabilidad disminuida (el 50% de los ratones Terc$^{-/-}$ de generaciones tardías en fondo BL6 mueren a los cinco meses de edad). Las generaciones tardías de ratones deficientes en telomerasa exhiben una resistencia, y esto es de importancia, cuando se someten a un protocolo de carcinogénesis multi-estadío usando 7,12-dimetilbenz(a)antraceno (DMBA) y 12-O-tetradecanoilforbol 13-acetato (TPA) [55]. Los ratones deficientes en telomerasa brindan un modelo excelente para el estudio de la implicación de la telomerasa y los telómeros en la tumorigénesis y el envejecimiento.

Se cree que la telomerasa es la responsable del establecimiento de una homeostasis normal de la longitud telomérica en los compartimentos de las células madre de los tejidos adultos [31, 34, 35, 56, 57]. Se encontró un número elevado de células madre en la epidermis y los folículos pilosos en las generaciones tardías de ratones deficientes en telomerasa, mientras el número de células madre era menor en los ratones suficientes en telomerasa. Estos hallazgos se pueden explicar mediante defectos en la movilización de las células madre relacionados con la deficiencia en telomerasa. Consecuencia de lo anterior son una cicatrización disminuida, un aclaramiento del cabello y una menor incidencia del cáncer, procesos relacionados con deficiencias en la regeneración tisular [25, 51, 52, 55]. Basado en conocimiento adquirido a partir del estudio de los modelos murinos, el acortamiento de los telómeros en el contexto del proceso de envejecimiento de los tejidos humanos afecta a la homeostasis tisular porque impide la movilización de las células madre adultas. Por el contrario, la sobre-expresión de la telomerasa en los ratones transgénicos para Tert resulta en un aumento de la movilización de las células madre [34]. Esto conduce a un incremento de la proliferación celular, un mayor grosor de la piel y a que los queratinocitos tengan un elevado potencial de formación de colonias, lo cual resulta en un estado de forma tisular mejorado.

7.2.2. Células madre

Las células madre, son células no especializadas que pueden originar diferentes tipos de células, poseen la habilidad de dividirse y renovarse a sí mismas a lo lago de la vida de un órgano. Se pueden agrupar en células madre embrionarias y células madre adultas. Las células madre embrionarias son pluripotentes, lo que significa que pueden convertirse en todos los tipos celulares del cuerpo y tienen incluso el potencial para generar un organismo completo. Las células madre adultas son multipotentes,

son indiferenciadas y se emplazan en tejidos donde son responsables del mantenimiento a largo plazo y la regeneración del tejido.

7.2.2.1. Los nichos de las células madre

Se define el nicho de las células madre como el microentorno celular que satisface todos los requerimientos para el mantenimiento de dichas células. Los nichos ofrecen protección frente a varios estímulos que podrían conducir a la diferenciación o a la apoptosis, tienen que coordinar la quiescencia y la actividad de las células madre, y regular su producción para evitar una proliferación celular descontrolada que pueda conducir a la formación de un tumor [58]. Las células madre tienen que producir constantemente las células progenitoras o las amplificadoras de tránsito (TA). Estas células TA tienen la habilidad de diferenciarse y generar el tejido correspondiente. La diferenciación de estas células se puede inducir por ciertos estímulos, tales como las heridas. Aunque se evita la expansión, las reservas de células madre se rellenan continuamente, este mecanismo confiere al organismo protección frente a la formación de tumores [59]. Entre los nichos de células madre que han sido bien caracterizados se encuentran los del intestino, los hematopoyéticos y los de los folículos pilosos de la epidermis.

7.2.3. Telómeros, células madre, envejecimiento y cáncer

Puesto que el daño celular contribuye a la tumorigenesis y al envejecimiento, los mecanismos protectores frente a este daño proporcionan una cobertura tanto frente al cáncer como al envejecimiento [60]. Entre los mecanismos que protegen frente a la formación de tumores se encuentra el de la limitación del potencial proliferativo de las células, que, como contrapartida, puede conducir a un envejecimiento acusado.

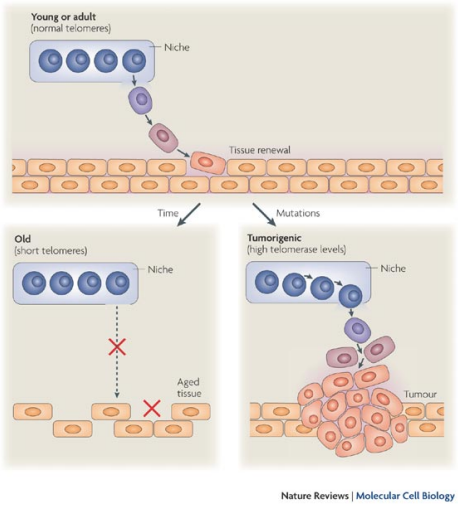

Figura 5: Un modelo para la implicación de los telómeros y la telomerasa en la biología de las células madre (se describe en amplio detalle en el texto). Los círculos azules representan células madre, los cuadrados naranjas células diferenciadas [60].

La figura 5 ilustra un modelo para explicar la movilización celular y la regeneración tisular en los procesos de cáncer y envejecimiento. Los organismo jóvenes o adultos con una longitud telomérica normal cuentan con células madre funcionales que, consecuentemente, pueden regenerar los tejidos. Se ha presentado la hipótesis de que en los organismos envejecidos (abajo a la izquierda) el potencial proliferativo está afectado debido a un acortamiento crítico de los telómeros de las células madre. Las células madre, subsiguientemente, no pueden abandonar sus nichos y los tejidos no se regeneran de forma eficiente, lo que ocasiona que el órgano falle. La baja movilización de las células madre puede tener un efecto positivo, como puede ser la minimización de la formación de tumores causados por una

elevada proliferación (abajo a la derecha). Los organismos que expresan un nivel de telomerasa alto disfrutan de un potencial de movilización de células madre aumentado, de una mayor regeneración tisular y por tanto de una mejor forma. Sin embargo, el tiempo de vida potencialmente más elevado, se ve afectado de forma negativa por un mayor riego de desarrollar cáncer [60]. Los defectos múltiples en los órganos son característicos del envejecimiento dependiente de los telómeros y una consecuencia del fallo en las células madre que causa los defectos en la regeneración tisular [18].

7.2.4. Los telómeros y los defectos en las células madre

Hay un número de enfermedades humanas que se sabe están asociadas a la pérdida de la telomerasa en las células madre. Por ejemplo, la disqueratosis congénita (DKC) está causada por un defecto en el gen que codifica para la molécula de ARN que la telomerasa emplea como molde, lo cual resulta en un acortamiento telomérico prematuro. Algunos de los síntomas típicos de los pacientes con DKC son fallos en la médula ósea, trastornos intestinales o tumores malignos antes de alcanzar los 50 años de edad. En los pacientes con DKC ligada al cromosoma X se visto que el gen DKC1 (que codifica a la proteína disquerina) es defectuoso. La disquerina forma un complejo con la telomerasa, lo que presumiblemente afecta a la actividad de la enzima [5]. Se pudo identificar que el gen DKC1 está involucrado en esta enfermedad gracias al estudio de una familia con una deleción 3' autosómica dominante en este gen. La DKC se caracteriza por la ocurrencia de anemia aplásica, un trastorno de la médula ósea que, en esta enfermedad, hace que contenga mucha grasa y, en cambio, un escaso número de células hematopoyéticas. Los enfermos con anemia aplásica presentan mutaciones en Terc y Tert en las líneas germinales [8, 61]. La inestabilidad genómica, adicionalmente, puede causar desarrollo de tumores [63, 64]. Y se ha visto que la susceptibilidad al desarrollo de cáncer en los pacientes de DKC es muy elevada, sucede en un 10% de los casos [62]. Los tejidos con una alta capacidad regenerativa son, notablemente, especialmente proclives a la formación de tumores. En resumen, el fallo en las células madre es una consecuencia del acortamiento telomérico y se observa de manera prominente en los tejidos con alta capacidad de regeneración, como los de la piel y la médula ósea.

7.2.5. ¿Pueden curarse los defectos en las células madre con la restauración de la actividad telomerasa?

La re-introducción de la actividad telomerasa puede ser una aproximación prometedora al tratamiento de las enfermedades relacionadas con la edad y causadas por un acortamiento crítico de los telómeros. Esta idea ya se ha examinado en el modelo murino deficiente en telomerasa. Se cruzaron ratones que carecen de telomerasa de generaciones tardías, G3 $Terc^{-/-}$, que tienen telómeros cortos y defectos proliferativos severos, con ratones $Terc^{+/-}$. Este cruce produjo una progenie de generación tardía deficiente en telomerasa, G4 $Terc^{-/-}$ que contenía un conjunto de cromosomas con telómeros de longitud normal proveniente del progenitor $Terc^{+/-}$. El otro conjunto de cromosomas retenía los telómeros críticamente cortos provenientes del progenitor G3 $Terc^{-/-}$. Todos los cromosomas de la camada, reconstituidos con un alelo del gen terc, $Terc^{+/-}$, tenían telómeros detectables [65]. Los

telómeros críticamente cortos se alargaron en aquellos ratones en los que se habia reconstituido la actividad telomerasa, se rescató la inestabilidad cromosómica y se previnieron los defectos proliferativos severos [65]. No se ha investigado de manera exhaustiva, sin embargo, el efecto que pueda tener la re-introducción de la telomerasa sobre el comportamiento de las células madre. La homeostasis y la supervivencia de los órganos son consecuencia de la capacidad de las células madre para regenerar el tejido correspondiente. Este proyecto se planteó con el propósito de estudiar si la restauración de la actividad telomerasa sería suficiente para atenuar los defectos en las células madre. Así pues, se reintrodujo la telomerasa en ratones deficientes en telomerasa de generación tardía y se investigó la biología de las células madre de la epidermis, que es un modelo muy bien establecido para la exploración de la biología de las células madre.

7.2.6. Las células madre adultas de la piel y el folículo piloso

Se han identificado dos poblaciones de células madre con capacidad para la regeneración de la capa epidérmica y sus tejidos asociados dentro de los folículos pilosos y las regiones interfoliculares. Las capas de la piel se regeneran mediante las células madre residentes en la membrana basal de la piel. Las células madre del folículo piloso (HFSC) se localizan en el bulbo capilar y son las responsables de la renovación del pelo y la glándula sebácea [66].

7.2.6.1. La piel

La Figura 6 muestra los componentes dérmicos y epidérmicos de la piel de los mamíferos. La proliferación ocurre en la capa basal de la epidermis. Las células se diferencian gradualmente y emigran hasta que alcanzan la capa superior, que se conoce con el nombre de *capa córnea*. Las células provenientes de la capa basilar entran en la capa espinada, donde pierden su actividad mitótica pero, a cambio, contribuyen al endurecimiento y solidificación del epitelio, las queratinas que producen estas células mantienen este proceso. Las células se desplazan hacia la superficie cutánea y tras una diferenciación terminal experimentan una "muerte celular programada" similar a la apoptosis normal [67]. La capa córnea contiene las escamas planas muertas que se reemplazan continuamente por células internas que se diferencian hacia afuera. Se garantiza de esta manera una barrera efectiva frente a las influencias dañinas provenientes del exterior así como una protección de los fluidos y tejidos del interior del organismo. En la piel de los ratones la diferenciación desde la capa basal a las escamas requiere de 10 a 14 días [68]. Se pensaba inicialmente que las células madres estaban presentes en la totalidad de la capa basal, pero tras un examen más detallado se estableció que solamente un 10-12% de las células eran posibles células madre [69].

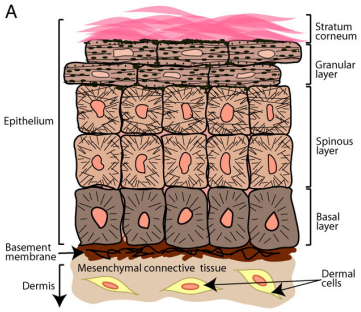

Figura 6: Histología del epitelio cutáneo. Se muestran las diferentes capas del epitelio (en el texto se expone una descripción más detallada) [58, 66].

7.2.6.2. El folículo piloso

La Figura 7 ilustra la estructura del folículo piloso, el cual es circundado por células de la dermis. Las células madre multipotentes se emplazan en el bulbo por debajo de la glándula sebácea. Se piensa que estas células producen los linajes del folículo piloso, la glándula sebácea, y la epidermis. La matriz del folículo piloso se sitúa alrededor de la papila dérmica y consiste en células amplificadoras transitorias que se diferencian para dar lugar a la vaina interna de la raíz y el tallo capilar [66]. Dentro del ciclo capilar las HFSC se aproximan continuamente a las papilas dérmicas para formar el cabello naciente [70]. El ciclo capilar consta de varias fases: de crecimiento (anágena), de involución (catágena) y de descanso (telógena). En la fase anágena se construye un cabello entero, en la fase catágena ocurre la apoptosis y la proliferación disminuye, y en la fase telógena no se detectan ni apoptosis ni proliferación [71].

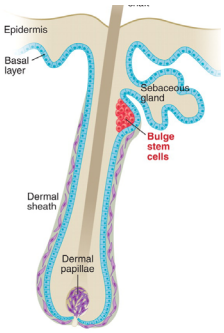

Figura 7: Histología del folículo piloso con su nicho [58].

7.3. Mecanismos moleculares de la función de los telómeros y la telomerasa en cáncer y envejecimiento

7.3.1. Señalización de daño en el ADN motivado por los telómeros cortos

Los telómeros representan roturas de ADN bicatenario (DSBs) con una hebra de ADN monocatenario (ssADN) adicional en el extremo. En los extremos de los cromosomas de los mamíferos se encuentran varias proteínas de reparación del daño al ADN. Estas proteínas en este caso no ejercen funciones de reparación del ADN, de parada de ciclo celular o de apoptosis como harían normalmente, sino que se encuentran incorporadas en un complejo nucleoproteico denominado *shelterin* que estabiliza y protege al telómero [11].

Como se mencionó en el capítulo 7.1.1, los telómeros se acortan tras cada división celular, como consecuencia del problema de la replicación de los extremos. La longitud telomérica se ve afectada por una replicación del ADN incompleta, el corte de las nucleasas en los extremos de los cromosomas, y la ausencia de actividad de la telomerasa [72]. Cuando los telómeros no pueden cumplir con su función protectora, como sucede cuando son críticamente cortos, las células entran en senescencia reflejando una respuesta de punto de control de daño en el ADN [73]. Los telómeros largos están protegidos por los complejos de *shelterin* los cuales se componen de tres subunidades, TRF1, TRF2 y POT1, y estas subunidades se conectan entre ellas mediante TIN2, TPP1, y Rap1 [1]. Los telómeros cortos han perdido este complejo protector y la maquinaria de reparación del ADN los reconoce como si fuesen un ADN dañado. Se activan entonces unas proteínas importantes que median la respuesta al daño en el ADN en eucariotas, tales como las kinasas conocidas como ATM (mutada de ataxia

telangiectasa) y ATR (Atm y Rad3 relacionada) [73]. Se ha demostrado que TRF2 previene la respuesta al daño en el ADN mediada por ATM, mientras que POT1 evita la activación de ATR [74].

7.3.1.1. La "hipótesis cáncer-envejecimiento"

La así denominada "hipótesis cáncer-envejecimiento" explica el efecto de los mecanismos que previenen el envejecimiento pero, en cambio, aumentan el nivel de tumorigénesis; viceversa, en los tejidos envejecidos la incidencia del crecimiento tumoral sería menor.

Los ratones deficientes en telomerasa y en los supresores tumorales constituyen una herramienta fundamental para el estudio del cáncer y el envejecimiento. Ha sido demostrado previamente que los ratones deficientes en telomerasa y con defectos en una proteína supresora de tumores presentan una tumorigénesis disminuida debido al efecto de supresor tumoral de los telómeros cortos, que provocan una parada del ciclo celular y apoptosis [75-82]. Es importante notar que los ratones que portan una mutación en p53 y sobre-expresan TRF2 (cuyos telómeros son cortos) han de ser excluidos, porque en estos ratones la incidencia del cáncer es incluso superior a la de los ratones silvestres (Fig. 8) [42, 83, 84]. Las patologías degenerativas que causa la deficiencia en telomerasa están atenuadas en los ratones que tienen un fondo deficiente en p53 así como en p21 [78, 83, 84]. La diferencia en los resultatos obtenidos para p53 y p21 se explican en función de la observación que indica que en los ratones con telómeros críticamente cortos la eliminación de p21 da lugar a una recuperación de la proliferación pero no de la apoptosis [29].

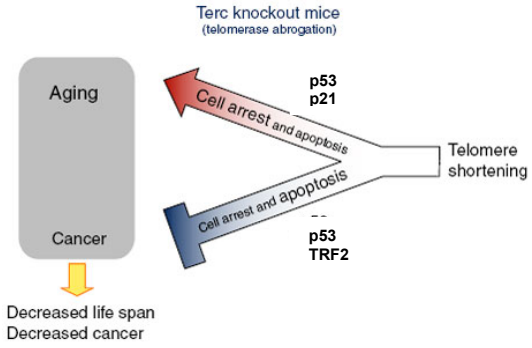

Figura 8: Acortamiento telomérico y p53, p21 y TRF2 (explicación detallada en el texto) (modificado de [29]).

Los telómeros no funcionales, el daño en el ADN, la activación de los oncogenes y las especies reactivas de oxígeno (ROS) inducen la ruta de p53, que resulta en apoptosis o en senescencia, dos reconocidos mecanismos para la supresión tumoral que también están involucrados en la respuesta al estrés (Fig. 9a) [85]. El inhibidor de las ciclinas dependientes de quinasas p21 es una diana de p53 y

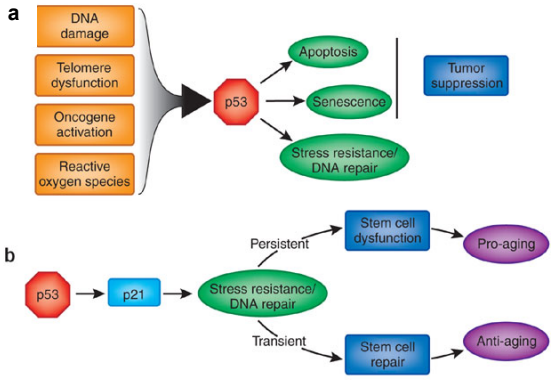

Figura 9: La función de p21 en la respuesta de p53 (a) p53 responde a varios estímulos y provoca la inducción de senescencia, apoptosis o una respuesta a daño en el ADN o estrés. La apoptosis y la senescencia se han vinculado a la supresión tumoral, mientras que aun no se ha establecido con claridad el papel de la respuesta a estrés en la prevención del cáncer. (b) p21 es un efector de p53 y parece inducir una parada reversible en el ciclo celular, permitiendo la reparación del ADN o la resistencia al estrés. Esta parada puede tanto proteger como comprometer la funcionalidad de las células madre y el envejecimiento dependiendo de la persistencia de un estímulo activador de p53 [85].

conduce a la parada del ciclo celular. Es importante notar que p21 no parece ser responsable de las propiedades de supresión tumoral de p53, aunque parece tener un rol destacado en la respuesta celular al daño en el ADN y en otras situaciones de estrés [78]. Una señal persistente de estrés tendría un efecto pro-envejecimiento en las células madre debido a la función de p21, mientras que una señal de estrés que fuese solo transitoria provocaría una reparación en la funcionalidad de las células madre y tendría, por tanto, un efecto anti-envejecimiento (Fig. 9b). Una aproximación prometedora sería la separación de las propiedades de promoción del envejecimiento y las de supresión tumoral de p53 con la ayuda de p21 [86]. Como consecuencia de la inhibición de p21, el proceso de envejecimiento se detendría o al menos se retrasaría, mientras que las actividades de supresión de tumores de p53 permanecerían intactas [85].

7.3.2. Telómeros – proteínas

Las proteínas que se asocian a los telómeros se pueden clasificar en aquellas de unión directa a la región de TTAGGG de doble hebra, y otras que se unen a la hebra-G monocatenaria o se unen indirectamente. TRF1 y TRF2 [87] pertenecen al primer grupo, mientras que proteínas como TIN2 [88], Tank1 y 2 [89-91], PINX1 [92], PTOP/PIP1 [93], el complejo Mre11 [94], el complejo DNA-PK [95], y PARP2 [96] pertenecen al último grupo.

7.3.2.1. TRF1 regula la longitud del telómero

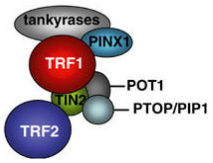

Figura 10: Los componentes del complejo del factor 1 de unión a las repeticiones teloméricas (TRF1) [18].

La proteína TRF1 (factor de unión a la repetición telomérica 1) está involucrada en el control de la longitud telomérica. Forma un complejo con otras proteínas entre las que se encuentran TIN2 (factor nuclear de interacción con TRF1) [88], las poli(ADP)-ribosilasas TANK1 TANK 2 (tanquirasas 1 y 2) [90, 97], POT1 (protección de la telomerasa 1), PTOP/PIP1 (proteína organizadora de POT1 y TIN2), y RAP1 (proteína represora/activadora 1). TRF1 y TRF2 se conectan indirectamente a través

de TIN2 [18] (Fig. 10). Cabe observar que una deleción dirigida de TRF1 no provoca cambios ni en la longitud telomérica ni en la protección del telómero. La deleción de esta proteína es, sin embargo, letal en embriones [98]. Una explicación a los fenotipos anteriores es que las proteínas que interaccionan con TRF1 compensan la falta de funcionalidad de TRF1. TANK1 es una de las proteínas que interaccionan con TRF1 y, adicionalmente, está involucrada en la división celular. La disminución de TANK1 origina la separación de las cromátidas hermanas en los centrómeros y los brazos, pero las cromátidas hermanas permanecen asociadas a los telómeros provocando una parada mitótica [99]. La ADP-ribosilación de TRF1 por TANK y TANK2 disminuye su capacidad de unión al ADN telomérico *in vitro* [90]. La inhibición de TRF1 por TANK1 está controlada por TIN2. La disminución parcial de TIN2 resulta en un alargamiento telomérico [100] mientras que los ratones que tienen inactivado el gen TIN2 mueren en el estado embrionario [101]. TIN2 se asocia con HP1γ, lo que apoya la hipótesis de que los dominios de TIN2 promueven la compactación de la cromatina [102]. TIN2 interacciona con TRF1 y TRF2 lo que sugiere que las funciones de ambas proteínas están conectadas por TIN2 [100, 103, 104]. TRF1 interacciona con la proteína POT1, transmitiendo así la información acerca de la longitud telomérica a los extremos de los telómeros, constituidos por la hebra-G monocatenaria protuberante, y en ultimo termino se regula la telomerasa [105]. POT1 se une al ADN telomérico monocatenario; es reclutada al telómero mediante la formación de un heterodímero con PTOP [93, 106, 107]. PINX1 (la proteína X-1 de interacción con PIN2/TRF1) es otra proteína de interacción con TRF1 que inhibe la actividad de la telomerasa y afecta a la tumorogenicidad, funcionando pues como un supresor de tumores putativo [108]. Cabe resatar que en algunos tipos de tumores humanos la expresión de TRF1, TRF2, TIN2, POT1 y TANK1 está alterada [109-113].

7.3.2.2. TRF2 protege y regula el telómero

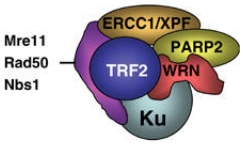

Figura 11: Los componentes del factor 2 de unión a las repeticiones teloméricas (TRF2) [18].

TRF2 está involucrada en la protección y la regulación de la longitud del telómero (Fig.11) [18]. TRF2 tiene una función clave en la protección de los extremos de los cromosomas para que no se formen fusiones entre dos extremos y para evitar la pérdida de la hebra-G protuberante [114]. Se ha visto que los niveles de TRF2 están alterados en algunos tumores [109, 113]. La sobre-expresión de TRF2 resulta en un fenotipo cutáneo en el ratón similar a la enfermedad *Xeroderma pigmentosum* que occure en humanos. Estos ratones exhiben una reacción severa cuando se exponen a la luz diurna [115]. Los queratinocitos que sobre-expresan TRF2 presentan un aumento de la inestabilidad cromosómica asociada a los telómeros muy cortos y a la pérdida de la hebra-G protuberante. Es más, la pérdida de los telómeros en los ratones que sobre-expresan TRF2 está mediada por XPF/ERCC1, una nucleasa implicada en la reparación del daño inducido en el ADN por la radiación ultravioleta mediante la reparación de la escisión de nucleótidos [115]. XPF/ERCC1 corta y elimina el extremo 3' protuberante de los telómeros tras la inhibición de TRF2 [116]. Las fusiones entre extremos, provocadas por la

unión de extremos no homólogos, son unas aberraciones típicas relacionadas con los telómeros no funcionales [117, 118]. Otro tipo de aberraciones cromosómicas, las translocaciones, son consecuencia de la unión de telómeros no funcionales a las roturas de la cadena doble [119]. Notablemente, TRF2 tiene una función en el establecimiento de una estructura adecuada del telómero, lo que indica que TRF2 puede convertir telómeros modelo en estructuras de *t-loop* [120]. TRF2 recluta a hRAP al telómero y regula negativamente su longitud [121, 122].

Es interesante observar que, varias de las proteínas reclutadas por TRF2 están relacionadas con el proceso de reparación del ADN. Por ejemplo, PARP2 que es importante en la reparación por escisión de bases y otras rutas de reparación, se une físicamente a TRF2 y funciona como un regulador negativo para TRF2 [123]. Se ha demostrado la existencia de una interacción entre Ku70 y TRF2 en células humanas mediante estudios de coinmunoprecipitación [124]. Las líneas celulares humanas que carecen de actividad telomerasa exhiben cuerpos PML asociados a ALT (APBs) que contienen, las proteínas TRF1, TRF2 y WNR, entre otras [125]. Los ABPs contienen también el factor de replicación A (RPA), Rad51, y Rad52, todos implicados en la síntesis y la recombinación del ADN [41]. También, el complejo Mer11, constituido por Rad50, Mre11 y NBS1 es importante en la recombinación homóloga (HR), en las rutas de unión de extremos no homólogos (NHEJ) y la reparación de las rotura de la doble hebra del ADN (DSB), por otro parte, también interacciona con TRF2 [94].

TRF2 es requerido para prevenir que los telómeros activen los puntos de control de daño en el ADN. Esta proteína se une específicamente a ATM y altera la respuesta al daño en el ADN que es dependiente de ATM. Se propuso así que TRF2 inhibia la activación de ATM en los telómeros [126]. La inhibición de TRF2 o el acortamiento telomérico resulta en la asociación de los telómeros con ATM y otros factores de la respuesta al daño en el ADN como la proteína 1 de unión a p53 (53BP1) y la fosforilación de γ-H2AX [73, 127, 128].

7.3.3. Daño en el ADN – Rutas del daño en el ADN y señalización

La preservación de un genoma intacto es fundamental para la vida de un organismo. Incluso el más pequeño de los daños en el ADN provoca una cascada de señalización que resulta en la reparación del ADN [129]. El daño en el ADN dispara la reparación de éste, la activación de los puntos de control del ADN, la respuesta transcripcional, y la apoptosis [130].

Los genes de la reparación del daño en el ADN se pueden subagrupar en genes asociados a la señalización y la reparación del daño en el ADN y los genes asociados a los diferentes mecanismos de reparación. Las rutas de reparación del daño en el ADN se pueden clasificar en rutas de reparación por escisión y rutas de reparación de roturas en la doble hebra.

Las rutas de reparación por escisión se pueden subdividir **en reparación por escisión de nucleótidos**, **reparación por escisión de bases** (la reparación de roturas en cadena sencilla se consideran a menudo parte de la reparación por escisión de bases) y **reparación de apareamientos erróneos** (descrito en más detalle en la sección 3.3.3.2.). En general, estas rutas operan mediante el reconocimiento del daño, escisión de la lesión por incisión en el esqueleto fosfodiéster del ADN, resíntesis de la sección que falta mediante la polimerasa de ADN (empleando como molde la cadena complementaria) y, por último, cierre de la hebra mediante ligación [131].

Numerosos agentes causan roturas en la doble hebra, como ciertos agentes químicos y la radiación ionizante, esta forma de daño es muy perjudicial para el organismo. **Las rutas de reparación de las roturas en la doble hebra** se pueden dividir en las de **unión de extremos no homólogos (NHEJ)** y **recombinación homóloga (HR)**. El NHEJ es responsable de la ligación de dos extremos de ADN roto que no tienen homología o con homología mínima. Este sistema de reparación es proclive a los errores debido a la ausencia de un molde intacto. Por el contrario, en el HR una secuencia homóloga sirve de molde y esta maquinaria de reparación está libre de error [131].

7.3.3.1. Puntos de control en el daño del ADN

Los puntos de control del ciclo celular aseguran la fidelidad de la división celular en las células eucariotas. Los puntos de control detienen el ciclo celular de forma específica durante un tiempo determinado para permitir la reparación y prevenir la replicación del ADN dañado, evitando por tanto la formación de cáncer. Los puntos de control de daño en el ADN son rutas de transducción de señales activadas por el daño en el ADN y son las responsables de los cambios en el ciclo celular, la regulación de los genes de reparación de daño en el ADN, la inducción de la transcripción y, cuando las células no pueden tolerar más daño, el disparo de las rutas de muerte celular apoptótica [132].

Los puntos de control de daño en el ADN se pueden agrupar, de forma amplia, en tres categorías: **sensores, transductores de señal, y efectores** (Fig. 12). Es importante señalar que no existe una separación clara porque ciertas proteínas pueden asumir las funciones de varios componentes.

Entre los **sensores** al daño en el ADN se han identificado dos quinasas de punto de control del tipo de la quinasa fosfoinosítido 3 (PI3K-like). Para cada uno de estos dos puntos de control se necesitan ATM ó ATR [133].

ATM es importante en la respuesta a la radiación ionizante (IR) y se cree que se activa principalmente en respuesta a las roturas en la cadena doble. ATM fosfoforila a muchas proteínas, como Chk2, p53

[134, 135], NBS1 [136], BRCA1 [137] y a sí misma [138]. Por otro lado, ATR se activa por la luz ultravioleta, los agentes de mutilación del ADN e inhibidores de la replicación como diroxiurea (HU) y afidicolina [139]. La reparación del ADN a través de esta vía suele resultar en regiones extendidas de ssADN. ART fosforila casi todas las proteínas que fosforila ATM y funciona mediante la formación de un complejo con ATRIP (proteína de interacción con ATR). Adicionalmente, se ha descrito que ATRIP percibe el daño en el ADN y media la activación de ATR mediante el reconocimiento de complejos RPA-ssADN [140].

Otro sensor del daño en el ADN es el complejo 9-1-1 (rad9-Rad1-Hus1), un homotrímero de estructura en forma de anillo que comparte una similitud de secuencia con PCNA, el factor de procesividad de la polimerasa de ADN. PCNA rodea al ADN y se une la polimerasa de ADN δ para aumentar su procesividad. Debido a las similitudes estructurales, se sugirió que el complejo 9-1-1 desempeña la misma función que PCNA [141]. Por otro lado, Rad17 es similar a la subunidad mayor de RFC (factor de replicación C), un factor accesorio de PCNA [142]. Esta observación indica un papel de Rad17-RFC en la carga del complejo 9-1-1 sobre el ADN dañado [133, 143].

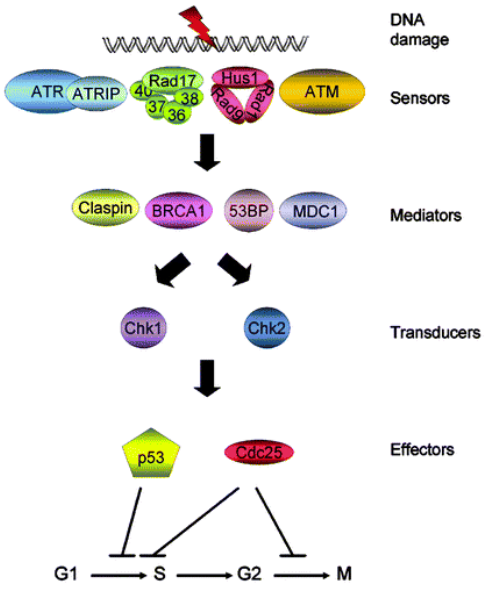

Figura 12: Componentes de los puntos de control de daño en el ADN en las células humanas. Los sensores detectan el daño, los mediadores translucen la señal a los transductores y los efectores participan directamente en ciertos puntos de control del ciclo celular [129].

La función de los **mediadores** es la de asociarse con los sensores de daño y los transductores de señal. ATM interacciona con proteínas mediadoras como la proteína de unión a p53, 53BP1 [144, 145], la proteína de unión a la topoisomerasa, TopBP1 [146], y el mediador del punto de control de daño en el ADN 1, MDC1 [147-149].

En las células humanas hay dos **transductores de señales**, las quinasas Chk1 y Chk2, que inhiben la progresión del ciclo celular [133, 150, 151]. En mamíferos, Chk2 se fosforila en una manera que depende de ATM y es inducida por la radiación ionizante [152, 153]. Al contrario, Chk1 se fosforila en respuesta a ATR [139, 154]. Las funciones de ambas proteínas, sin embargo, se solapan.

Las proteínas **efectoras** incluyen a la fosfatasa de fosfotirosina Cdc52 y p53. Estas proteínas habilitan la activación del punto de control mediante la regulación directa de importantes proteínas del ciclo celular en las transiciones, tanto de la fase G1 a la S como en la de la fase G2 a la M.

7.3.3.2. Reparación de apareamientos erróneos (MMR)

MMR es una ruta de reparación del ADN importante que satisface varias tareas:

- MMR corrige los apareamientos erróneos base-base y las inserciones/deleciones de *loops* durante la síntesis del ADN
- MMR está involucrada en la respuesta celular a varios tipos de daño en el ADN
- las proteínas MMR están involucradas en las recombinaciones mitóticas y meióticas
- las proteínas MMR desempeñan una función en la inestabilidad de las repeticiones de tripletes
- las proteínas MMR tienen una función en la generación de la diversidad de las inmunoglobulinas [155-157]

Los defectos en las rutas de reparación de los apareamientos erróneos desencadenan el desarrollo de los cánceres de colon no poliposos hereditarios, típicos y no típicos. Adicionalmente, los defectos en esta maquinaria de reparación son responsables de la ocurrencia del 15-25% de los tumores esporádicos [158, 159].

7.3.3.2.1. Mecanismo de reparación de apareamientos erróneos en los eucariotas

La maquinaria MMR reconoce los apareamientos erróneos base-base y las inserciones/deleciones de *loops* (IDLs) y los corrige en una manera libre de error. Degradan la secuencia que contiene el error en la cadena nueva que se ha sintetizado y la polimerasa de ADN puede crear una copia nueva empleando como molde el resto de la cadena.

La inestabilidad de los microsatélites es una consecuencia de los defectos en la reparación de los apareamientos erróneos. De forma característica, los microsatélites consisten en motivos de secuencias repetidas, como $[A]_n$ o $[CA]_n$. Estos motivos se distribuyen por todo el genoma y son la razón de la formación de las moléculas de heteroduplex de ADN, producidas por el anillamiento de las cadenas sencillas complementarias derivadas de moléculas duplex parentales diferentes durante la síntesis del ADN. Una secuencia microsatélite puede provocar la disociación entre un cebador y una cadena molde para generar un reanillamiento incorrecto. Las unidades de repetición de microsatélites en el molde y en la cadena de nueva síntesis difieren y originan la formación de ***loops* de inserción/deleción**.

Adicionalmente, los errores de la polimerasa de ADN que escapan a la corrección de pruebas son los responsables de la aparición de los **apareamientos erróneos base-base** [157]**.**

El mecanismo:

El inicio del proceso de reparación en células humanas es responsabilidad de tres proteínas. MSH2 y MSH6 forman el heterodímero **MutSα** y reconoce el apareamiento entre bases erróneo y los IDLs de una o los dos nucleótidos extrahelicoidales. **MutSβ** consta de MSH2 y MSH3 y se une a los IDLs de

mayor tamaño [155, 160-162]. Al igual que otras proteínas que participan en la reparación del ADN, las proteínas MSH son ATPasas con un motivo de unión a ATP de Walker [163, 164].

En las células humanas existen cuatro homólogos de **MutL:** MLH1, MLH3, PMS1 (proteína 1 de post-segregación meiótica) y PMS2. Estas proteínas son ATPasas de la familia de girasa/Hsp90/histidina-quinasa/Mult. (GHKL) [165] y forman tres heterodímeros diferentes. El complejo MLH1-PMS2 (MutLα) es el más importante. Hasta ahora, el mecanismo exacto de la reparación de los apareamientos erróneos no ha estado claro [157].

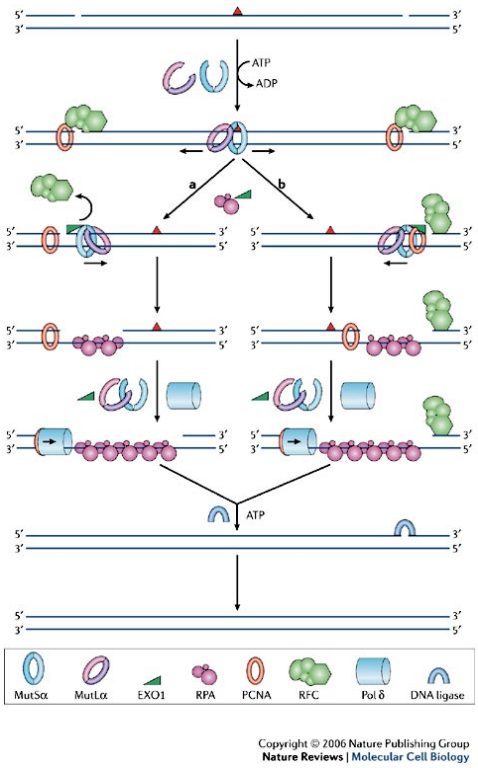

Figura 13: El sistema humano de reparación de apareamientos erróneos. Las proteínas involucradas en la reparación de apareamientos erróneos (MMR) son MutSα o MutSβ, MutLα, proteína de replicación A (RPA), exonucleasa 1 (EXO1), antígeno nuclear de proliferación nuclear (PCNA), factor de replicación C (RPC), polimerasa de ADN δ (Pol δ), y ligasa I de ADN (consultar el texto para mayor detalle) [157].

Numerosos estudios bioquímicos y estructurales han permitido elaborar el modelo [157] que se muestra en figura 13, en el cual MutSα o MutSβ reclutan a MutLα y el complejo comienza a difundirse desde la lesión en ambas direcciones [157].

Hacia arriba de la lesión, el RFC probablemente se reemplaza tras la carga de la exonucleasa 1 (EXO1) y EXO1 degrada la cadena en dirección 5' a 3'. La RPA es responsable de la estabilización del hueco en la cadena sencilla. MutSα por un lado, estimula la actividad de EXO1 mientras que MutLα, por otro, inhibe a EXO1 una vez que se ha eliminado el apareamiento erróneo. La polimerasa de ADN δ junto con PCNA rellena el hueco y la ligasa I de ADN conecta los extremos que quedan, finalizando de esta manera el proceso.

Hacia debajo de la lesión, se piensa que el complejo MutSα, MutLα, EXO1, PCNA y RFC (y posiblemente la caja 1 del grupo de alta movilidad (HMGB1)) sean los responsables de la degradación de la cadena que contiene el error. El ADN de cadena simple estaría protegido por RPA y la polimerasa de ADN δ podría llenar el hueco. De nuevo, la ligasa I de ADN finaliza la tarea conectando los extremos [157].

7.3.3.2.2. MMR en la señalización del daño en el ADN (figura 14)

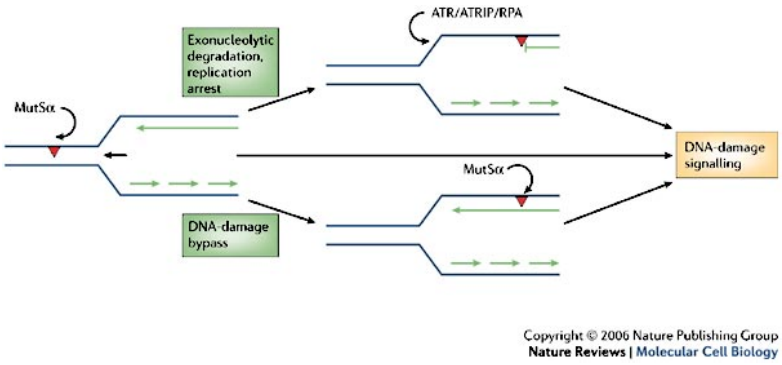

Figura 14: MMR en la señalización del daño al ADN: En este modelo, las proteínas de reparación de apareamientos erróneos (MutSa) pueden reconocer el daño al ADN (triángulo rojo), señalizar directamente a la maquinaria de control del ciclo celular e inducir la parada del ciclo celular y apoptosis (medio). El daño no puede ser procesado y la horquilla de replicación se detiene; las regiones del ADN de simple cadena reclutan la proteína de replicación A (RPA) y la proteína que interactúa con ATR (*ATR-interacting protein,* ATRIP), activan la quinasa ATR y la quinasa de puntos de control CHK1 (arriba). O, el daño puede ser ignorado por una ADN polimerasa propensa a errores y la señalización del daño al DNA sería producida por lesiones secundarias (abajo) [157].

Hay varias lesiones que provocan una respuesta de reparación del daño en el ADN como resultado de un proceso de reparación de apareamientos erróneos. Estas incluyen los metiladores de ADN S_N1, 6-tioguanina, 5-fluoro-desoxiuridina, cisplatina, luz ultravioleta, y varios carcinógenos. El procesamiento de estas lesiones por MMR no es efectivo y lleva a múltiples rondas de reparación fútiles, parada del ciclo celular y, finalmente, a apoptosis [156].

7.3.3.2.3. MMR y la recombinación

La recombinación homóloga (HR) implica el apareamiento de dos cadenas de ADN sencillo derivadas de diferentes dúplex parentales, para dar lugar a una molécula de ADN heterodúplex. Si las secuencias de las dos cadenas no son idénticas, el heterodúplex contendrá apareamientos erróneos. La principal consecuencia de los apareamientos erróneos en el ADN es la inestabilidad genómica a través de las translocaciones cromosómicas, las deleciones o las inversiones [166, 167].

El mecanismo de recombinación homóloga (Figura 15)

La HR comienza con la detección de un DSB, y el procesamiento de ambos extremos del ADN por una exonucleasa desconocida da lugar a un ADN monocatenario 3'. La RPA evita la formación de estructuras secundarias mediante su unión y protección al ADN de cadena sencilla. RPA es sustituida por Rad51, que se ayuda de Rad52, Rad55 y RAd57, y comienza la invasión de la cadena bicatenario homóloga. Las cadenas sencilla de ADN cambian de acompañante en un proceso que depende del ATP. Rad54 tiene una función muy importante aunque todavía no muy bien caracterizada. Es responsable, probablemente, de la apertura de la estructura de la cromatina para permitir la invasión de

la cadena y podría habilitar la liberación de Rad51 del ADN bicatenario. Se forma entonces un *loop*-D (loop de desplazamiento), que contiene ADN heteroduplex, y comienza la síntesis del ADN cebada por los extremos 3' de las cadenas sencillas de ADN. La HR finaliza el proceso mediante el corte del intermedio llamado cruce de Holliday y la ligación final [168].

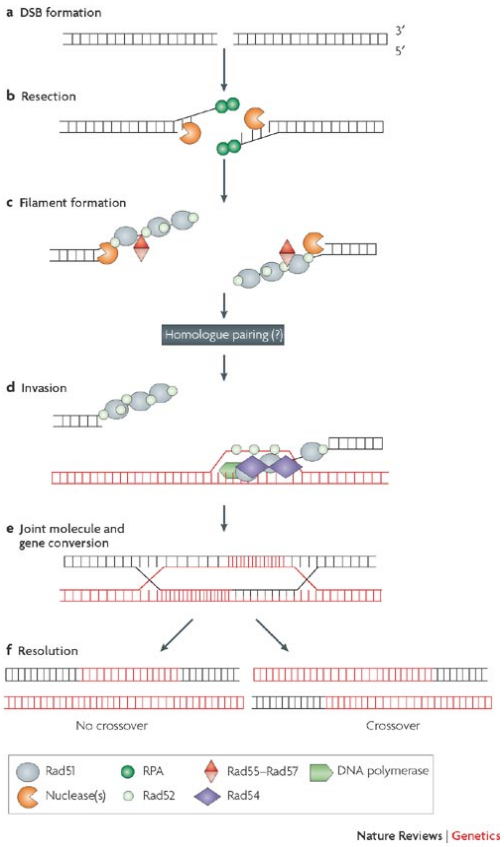

Figure 15: Homologous recombination (HR) in DSB-repair. The figure depicts the mechanism including the involved proteins (detailed description is provided in the text) [168].

La maquinaria de reparación de los errores de apareamiento participa en varios procesos relacionados con la HR. Pueden corregir los apareamientos erróneos, abortar la recombinación y eliminar las colas monocatenarias no homólogas [168].

La conversión de genes ocurre debido al MMR y a la actividad correctora de la polimerasa de ADN sobre los heteroduplex de los intermedios de recombinación. En este proceso se reemplaza la secuencia de uno de los cromosomas por una secuencia homóloga de otro cromosoma [169].

El sistema MMR de eucariotas tiene una **actividad anti-recombinatoria** que bloquea la recombinación entre secuencias divergentes en la mitosis y la meiosis [170]. Los estudios en las levaduras han demostrado que un único error en el apareamiento es suficiente para inhibir la recombinación entre secuencias por lo demás idénticas en una manera que es dependiente de la reparación de los errores de apareamiento. Los errores adicionales afectan a la tasa de recombinación en una manera acumulativa negativa hasta que se alcanza un nivel de divergencia tal que ya no se necesita la maquinaria de reparación de los errores de apareamiento para inhibir la recombinación [171].

Otra de las tareas de la maquinaria de MMR es la **eliminación de las colas no homólogas** que se generan en la recombinación homóloga. Una cola de ADN monocatenario con un extremo 3' invade una molécula de ADN dúplex homólogo y sirve como cebador para síntesis del ADN. Sin embargo, si el extremo 3' no es homólogo al dúplex que invade, el segmento no homólogo tiene que ser eliminado antes de que comience la síntesis del ADN [169]. Aunque las proteínas de MMR no desempeñan una función directa, se ha demostrado en las levaduras que MSH2 y MSH3 cooperan con la endonucleasa Rad1-Rad10 para eliminar las colas 3' [172, 173].

8. OBJECTIVES

8.1. To examine the impact of telomerase re-introduction on the stem cell biology and long-term survival of late generation telomerase-deficient mice

The mobilization of stem cells is a fundamental aspect of organ homeostasis and telomere length has been shown to play a critical role in this process. Telomerase deficiency leading to continuous telomere shortening until a critical length results in decreased epidermal stem cell mobilization and consequently defective tissue regeneration. In previous work it was found that the skin and the hair growth of late generation telomerase-deficient mice were impaired and, in agreement with the *in vivo* results, the *in vitro* proliferation capacity of the epidermal stem cells was decreased [34]. To further explore the link between stem cell biology and telomere-mediated aging we restored telomerase activity in late generation telomerase-deficient mice to asses whether this was sufficient to correct stem cell defects associated with short telomeres. The goal of this study was to determine if therapies based on telomerase activation would rectify the age-promoting effects of short telomeres and, importantly, if this would happen in the absence of an increased risk of carcinogenesis. These findings could be put into practice in the treatment of premature aging diseases, like dyskeratosis congenita [4-6], aplastic anemia [7, 8], and idiopathic pulmonary fibrosis [9, 10]. A common underlying cause of these diseases is decreased telomerase activity and shorter telomeres leading to impaired tissue regeneration and increased cancer incidence [174].

8.2. To study the relationship between mismatch repair proteins and telomeres

The object of this project was to explore the role of MMR proteins at mammalian telomeres, and their impacts on cancer and aging. Studies in yeast have shown that telomerase-deficient strains with a MMR defect do not exhibit the survival defect usually observed in cells that are telomerase-deficient alone [13]. It was postulated that increased usage of the ALT pathway was responsible for this observation, since ALT is recombination mediated, it is normally repressed by MMR. Bechter *et al.* (2004) described increased telomeric recombination in MMR-deficient human colon cancer cells [14]. Additionally, MMR proteins are involved in DNA damage signaling and this in turn could have an effect on tumor development [16, 17, 175]. To further deepen our understanding of the link between MMR proteins and telomere biology mice with short telomeres and mismatch repair defects were generated and characterized. Interestingly, we could not confirm a role of the MMR protein PMS2 in telomeric recombination, but, importantly we found that PMS2 and p21 may be in the same genetic pathway that has evolved to signal cell cycle arrest associated with telomeric dysfunction.

9. FIRST PROJECT

9.1. INTRODUCTION: Telomerase reverses epidermal hair follicle stem cell defects and loss of long-term survival associated with critically short telomeres

In contrast to a majority of somatic cells, telomerase is conspicuously active in stem cells suggesting a relationship between telomerase level and organismal fitness. Aplastic anemia [7, 8], dyskeratosis congenita [4-6], and idiopathic pulmonary fibrosis [9, 10] are consequences of mutations in either of the telomerase components Tert or Terc. These diseases are characterized by skin abnormalities and bone marrow failure due to defects in the stem cell compartments [174, 176, 177]. Similarly, telomerase-deficient mice with critically short telomeres show atrophies in skin, bone marrow, intestine and testis [25]. These observations suggest that telomere length and telomerase activity are important factors in stem cell biology and, therefore, also important determinants of organ homeostasis and organismal survival.

Flores et al. (2005) discovered a defect in the mobilization of hair follicle stem cells out of their stem cell niche in late generation telomerase-deficient mice leading to impaired regeneration of the hair and the skin [34]. These observations explain the premature skin-aging phenotype, decreased wound healing, hair loss, and hair graying of these mice [25, 51, 52]. Additionally, it was shown that late generation telomerase-deficient mice are resistant to skin carcinogenesis protocols [55].

Together, these observations raise an important question: Could the re-introduction of telomerase activity be sufficient to correct these stem cell defects and extend organismal life span?

To address this question we crossed late generation telomerase-deficient mice with normal heterozygous mice to restore one allele of the terc gene and investigated the consequences of this reconstitution.

Personal contribution:
I performed all experiments. Juana Flores analyzed the pathologies of the mice.

9.2. INTRODUCCIÓN: La telomerasa revierte los defectos de las células madre del folículo piloso epidérmico y la pérdida de la supervivencia a largo plazo asociada a los telómeros críticamente cortos

En contraste con la mayoría de las células somáticas, la telomerasa está notablemente activa en las células troncales, sugiriendo que existe una relación entre el nivel de telomerasa y el buen estado del organismo. La anemia aplásica [7, 8], la disqueratosis congénita [4-6] y la fibrosis pulmonar idiopática [9, 10] se producen como consecuencia de mutaciones en uno de los componentes de la telomerasa, Tert o Terc. Estas enfermedades se caracterizan por la ocurrencia de anormalidades en la piel y fallos en la médula ósea debido a defectos en los compartimentos de las células troncales [174, 176, 177]. De forma similar, los ratones deficientes en telomerasa, que tienen telómeros críticamente cortos, muestran atrofia en piel, médula ósea, intestinos y testículos [25]. Estas observaciones sugieren que la longitud telomérica y la actividad de telomerasa son factores importantes en la biología de las células troncales y, de esta forma, se constituyen en importantes determinantes de la homeostasis orgánica y la supervivencia del organismo.

Flores *et al*. (2005) descubrieron un defecto en la movilización de las células troncales hacia fuera de su nicho en ratones deficientes en telomerasa de generaciones tardías, conduciendo a una desmejorada regeneración del pelo y la piel [34]. Estas observaciones explican el fenotipo de envejecimiento prematuro de la piel, una disminución en la cicatrización de las heridas y pérdida del pelo y aparición de canas de estos ratones [25, 51, 52]. Además, también se ha demostrado que los ratones deficientes en telomerasa de generaciones tardías son más resistentes a los protocolos de carcinogénesis en piel [55].

A partir de estas observaciones en conjunto surge una importante pregunta: ¿La re-introducción de la actividad de telomerasa podría ser suficiente para corregir estos defectos en las células troncales y extender la vida media del organismo?

Para responderla, cruzamos ratones deficientes en telomerasa de generaciones tardías con ratones heterocigotos normales para restaurar un alelo del gen *terc* e investigamos las consecuencias de esta reconstitución.

Contribución personal:
Todos los experimentos han sido llevados a cabo por mí; Juana Flores ha analizado las patologías de los ratones.

9.3. PUBLICATION

Siegl-Cachedenier I, Flores I, Klatt P, Blasco MA.

Telomerase reverses epidermal hair follicle stem cell defects and loss of long-term survival associated with critically short telomeres.

J Cell Biol. 2007 Oct 22;179(2):277-90.

Telomerase reverses epidermal hair follicle stem cell defects and loss of long-term survival associated with critically short telomeres

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Organ homeostasis and organismal survival are related to the ability of stem cells to sustain tissue regeneration. As a consequence of accelerated telomerase shortening, telomerase-deficient mice show defective tissue regeneration and premature death. This suggests a direct impact of telomere length and telomerase activity on stem cell biology. We recently found that short telomeres impair the ability of epidermal stem cells to mobilize out of the hair follicle (HF) niche, resulting in impaired skin and hair growth and in the suppression of epidermal stem cell proliferative capacity in vitro. Here, we demonstrate that telomerase reintroduction in mice with critically short telomeres is sufficient to correct epidermal HF stem cell defects. Additionally, telomerase reintroduction into these mice results in a normal life span by preventing degenerative pathologies in the absence of increased tumorigenesis.

Introduction

Telomeres, which are composed of tandem repeats of the TTAGGG sequence and associated proteins, are nucleoprotein structures that cap the ends of chromosomes (for reviews see Blackburn, 2001; Chan and Blackburn, 2002; de Lange, 2005). Telomere length is maintained by telomerase, a reverse transcriptase that counteracts telomere shortening associated with cell division by de novo addition of telomere repeats onto chromosome ends (for review see Chan and Blackburn, 2002). Telomerase is expressed in the stem cell compartment of several adult tissues, where it is thought to compensate for telomere shortening associated with cell proliferation and tissue regeneration (for reviews see Collins and Mitchell, 2002; Harrington, 2004; Flores et al., 2005, 2006; Sarin et al., 2005). Interestingly, telomerase activity levels are not sufficient to maintain telomere length during human aging, and telomeres progressively shorten with increasing age in the context of the organism (Harley et al., 1990; Canela et al., 2007; for review see Blasco, 2005) and are also associated with different disease states (Samani et al., 2001; Wiemann et al., 2002; Cawthon et al., 2003; Epel et al., 2004; Valdes et al., 2005). Indeed, telomerase levels in humans and mice are thought to be rate limiting for organismal life span. In particular, reduced telomerase activity caused by mutations in telomerase components in the human diseases dyskeratosis congenita (Mitchell et al., 1999; Vulliamy et al., 2001, 2004; Mason et al., 2005), aplastic anemia (Marrone et al., 2004; Yamaguchi et al., 2005), and idiopathic pulmonary fibrosis (Armanios et al., 2007; Tsakiri et al., 2007) leads to accelerated telomere shortening, premature loss of tissue regeneration, and premature death. Some of these phenotypes are shared by telomerase-deficient mice ($Terc^{-/-}$ mice; Blasco et al., 1997), which show a reduction in both the median and the maximum life span already within the first mouse generation (García-Cao et al., 2006). Furthermore, these defects are anticipated with subsequent $Terc^{-/-}$ mouse generations with progressively shorter telomeres concomitant with premature loss of tissue regeneration and organismal survival (Lee et al., 1998; Herrera et al., 1999; Leri et al., 2003; Flores et al., 2005; García-Cao et al., 2006). Similarly, disease anticipation has also been reported with increasing generations of human dyskeratosis congenita patients (Vulliamy et al., 2004).

The telomerase-deficient mouse model has been instrumental in understanding the effects of telomere shortening on stem cell biology (for review see Blasco, 2005). In particular, we recently showed that telomere shortening results in an impaired capacity of hair follicle (HF) stem cells to regenerate the hair and the skin because of a defective mobilization of the HF stem cells out of their niche (Flores et al., 2005). This defective stem cell behavior anticipates the fact that telomerase-deficient mice show premature skin-aging phenotypes such as decreased

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Abbreviations used in this paper: a.u.f., arbitrary units of fluorescence; HF, hair follicle; IFE, interfollicular epidermis; LRC, label retaining cell; MEF, mouse embryonic fibroblast; Q-FISH, quantitative FISH; TPA, 12-O-tetradecanoylphorbol-13-acetate.

The online version of this article contains supplemental material.

The Journal of Cell Biology, Vol. 179, No. 2, October 22, 2007 277–290
http://www.jcb.org/cgi/doi/10.1083/jcb.200704141

Supplemental Material can be found at:
http://www.jcb.org/cgi/content/full/jcb.200704141/DC1

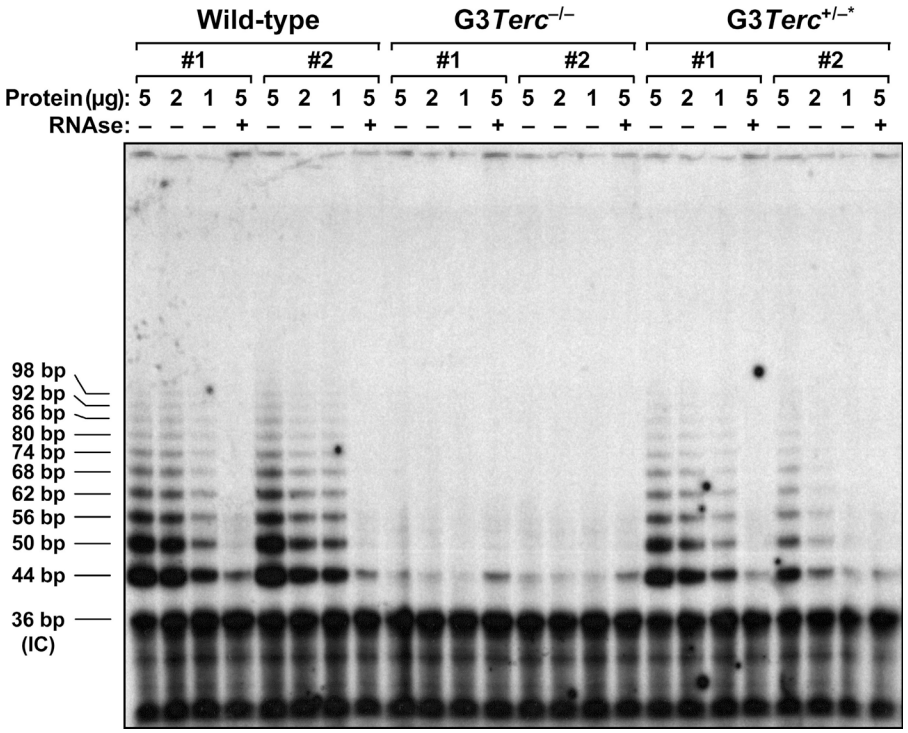


Figure 1. **Reconstitution of telomerase activity in G3 *Terc*$^{+/-*}$ mice compared with their G3 *Terc*$^{-/-}$ siblings.** Telomerase is successfully reconstituted in two independent MEF cultures from G3 *Terc*$^{+/-*}$ mice compared with their telomerase-deficient G3 *Terc*$^{-/-}$ littermates. Two wild-type MEFs are also included as a positive control for telomerase activity. The protein concentration used is indicated on top of each lane. Extracts were treated (+) or not treated (−) with RNase as a negative control. An internal control (IC) for PCR efficiency was also included.

wound healing, hair loss, and hair graying (Lee et al., 1998; Herrera et al., 1999; Rudolph et al., 1999), as well as decreased skin cancer, as indicated by the fact that they are resistant to skin carcinogenesis protocols (Gonzalez-Suarez et al., 2000). These findings suggested that the progressive telomere shortening that occurs in human tissues with increasing age might directly impact the ability of different adult stem cell populations to maintain tissue homeostasis. Furthermore, these results opened the possibility that restoration of telomerase activity may be sufficient to correct stem cell defects associated with short telomeres and to extend the organismal life span.

Here, we demonstrate that restoration of a copy of the Terc gene into late generation G3/G4 telomerase-deficient mice is sufficient to elongate critically short telomeres in skin keratinocytes from these mice, prevent end-to-end chromosome fusions, and rescue both HF stem cell defects in vivo and the impaired proliferative capacity of epidermal stem cells ex vivo. Finally, telomerase reintroduction was able to extend the normal life span of G4 telomerase-deficient mice by preventing degenerative pathologies in the absence of increased cancer. These findings support the notion that telomerase activators would be sufficient to correct stem cell defects in tissues with critically short telomeres in the absence of undesired effects.

Results

Reintroduction of the telomerase Terc gene in late generation Terc-deficient mice is sufficient to elongate critically short telomeres and prevent chromosomal instability in skin keratinocytes

To test whether telomerase reintroduction was able to elongate short telomeres in skin keratinocytes from late generation G3/G4 *Terc*-deficient mice, we first generated large colonies of littermate mice from *Terc*$^{+/-}$ × G2/G3 *Terc*$^{-/-}$ intercrosses (Materials and methods). The progeny of these crosses was divided into two mouse cohorts according to their telomerase status: G3/G4 *Terc*$^{-/-}$ telomerase-deficient mice and G3/G4 *Terc*$^{+/-}$ telomerase-reconstituted mice (referred to here as G3/G4 *Terc*$^{+/-*}$ mice). Importantly, both mouse cohorts inherited the same telomere length from the parents; however, G3/G4 *Terc*$^{-/-}$ mice lack telomerase activity and G3/G4 *Terc*$^{+/-*}$ mice are telomerase proficient (Fig. 1). To address whether telomerase activity in G3 *Terc*$^{+/-*}$ mice was able to rescue short telomeres compared with G3 *Terc*$^{-/-}$ cohorts, we measured telomere length using quantitative FISH (Q-FISH) on primary keratinocytes obtained from newborn mice (Gonzalez-Suarez et al., 2000; Muñoz et al., 2005; Materials and methods). G3 *Terc*$^{+/-*}$ skin keratinocytes showed, on average, longer telomeres than those from the corresponding G3 *Terc*$^{-/-}$ littermates ($P < 0.001$; Fig. 2, A and B). Furthermore, this telomere elongation coincided with a significant reduction of the percentage of short telomeres (<100 arbitrary units of fluorescence [a.u.f.]) in G3 *Terc*$^{+/-*}$ mice compared with the corresponding G3 *Terc*$^{-/-}$ littermates ($P < 0.001$; Fig. 2, A and B), which is in agreement with previous findings showing that telomerase preferentially elongates short telomeres both in yeast and mammals (Hemann et al., 2001; Samper et al., 2001; Teixeira et al., 2004). The percentage of longest telomeres (>1,000 a.u.f.) was also significantly increased in telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice compared with the corresponding G3 *Terc*$^{-/-}$ littermates ($P = 0.015$; Fig. 2, A and B), in agreement with the increase in mean telomere length.

Finally, we determined whether the elongated telomeres in G3 *Terc*$^{+/-*}$ keratinocytes correlated with a significant rescue of chromosomal aberrations associated with critically short telomeres. For this, we performed a full karyotypic analysis

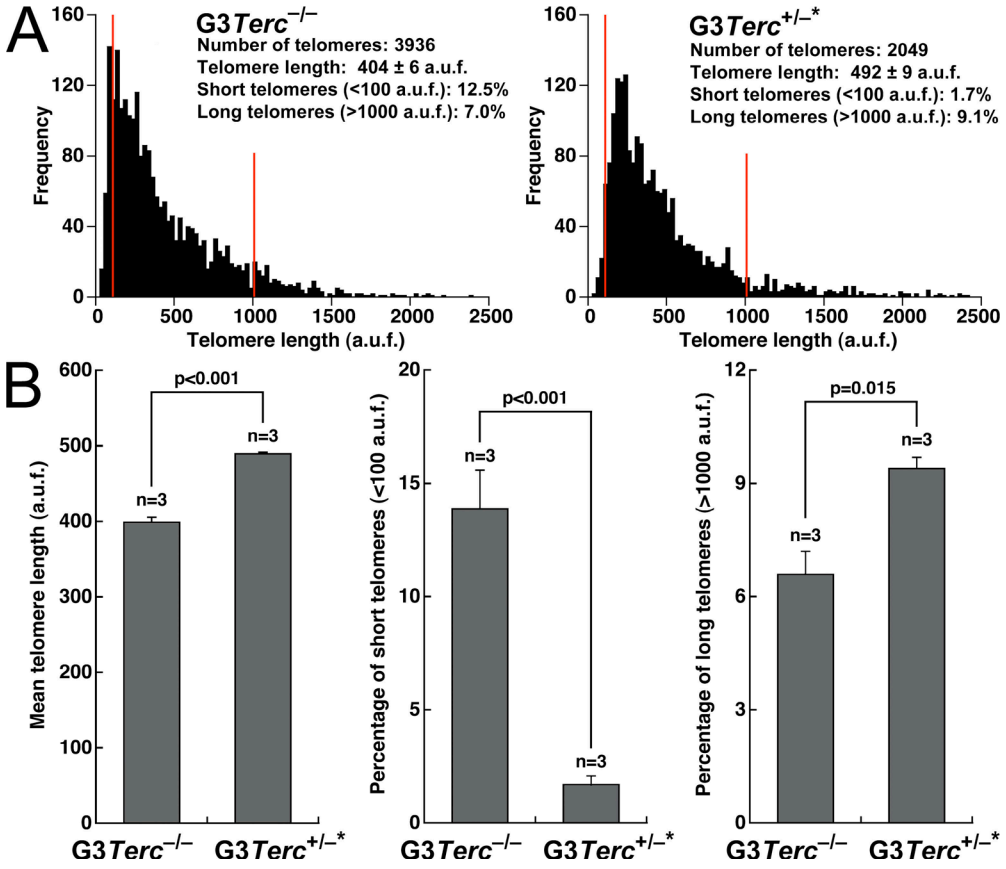


Figure 2. **Rescue of mean telomere length and percentage of short telomeres in late generation telomerase-reconstituted G3 *Terc*$^{+/-*}$ keratinocytes.** (A) Telomere length histogram obtained by Q-FISH in primary murine skin keratinocytes. The histogram shown is representative of three independent pairs of G3 *Terc*$^{-/-}$ and G3 *Terc*$^{+/-*}$ littermates (see B). Note a greater abundance of short telomeres in the G3 *Terc*$^{-/-}$ mouse compared with the G3 *Terc*$^{+/-*}$ littermate. Red lines facilitate visualization of short (<100 a.u.f.) and long (>1,000 a.u.f.) telomeres. The total number of telomere dots used for the quantification and the percentage of short and long telomeres are indicated. (B) Mean telomere length is significantly increased ($P < 0.001$) in G3 *Terc*$^{+/-*}$ compared with G3 *Terc*$^{-/-}$ mice. Concomitantly, these mice show a significant ($P < 0.001$) decrease in the percentage of short telomeres and a significant ($P = 0.015$) increase in the percentage of long telomeres. Data are mean values ± SEM for three independent pairs of G3 *Terc*$^{-/-}$ and G3 *Terc*$^{+/-*}$ littermates.

using telomere Q-FISH on metaphases (Materials and methods). As shown in Fig. 3 (A and B), telomerase-reconstituted G3 *Terc*$^{+/-*}$ keratinocytes showed a significant rescue of chromosomal abnormalities associated with critically short telomeres, such as signal-free ends and end-to-end fusions, compared with the corresponding G3 *Terc*$^{-/-}$ controls ($P < 0.001$ for all comparisons), suggesting that these types of aberrations are the direct consequence of critical telomere shortening and that when short telomeres are reelongated by telomerase they are completely prevented. In contrast, breaks and fragments are not rescued by telomerase reintroduction, indicating that they are not the direct consequence of critical telomere shortening.

Reintroduction of the telomerase Terc gene into G3/G4 Terc-deficient mice is sufficient to rescue mobilization defects of epidermal HF stem cells

To investigate whether elongation of short telomeres by telomerase in skin keratinocytes was sufficient to rescue epidermal stem cell defects in late generation telomerase-deficient mice (Flores et al., 2005), we compared the number and mobilization ability of epidermal stem cells in the HF stem cell niche before and after mitogenic activation in G3 *Terc*$^{+/-*}$ mice with that of the corresponding G3 *Terc*$^{-/-}$ littermates (Fig. 4, A–C). To visualize HF stem cells, we used a labeling technique previously shown to mark self-renewing and multipotent epidermal cells, the so-called label retaining cells (LRCs; for review see Fuchs et al., 2004; Flores et al., 2005; Moore and Lemischka, 2006). Of notice, these experiments were performed in young mice (0–2 mo old) from both genotypes before any skin phenotypes associated with short telomeres were detectable. Confocal microscopy revealed that LRCs are enriched at the bulge area of the HF in the two genotypes, which is in agreement with the known location of the HF stem cell niche (Fig. 4 C; Cotsarelis et al., 1990; Oshima et al., 2001; Morris et al., 2004; Tumbar et al., 2004).

In control resting skin conditions, we did not detect significant differences in the numbers of LRCs at the hair bulge of G3 *Terc*$^{+/-*}$ mice compared with the corresponding G3 *Terc*$^{-/-}$ littermates ($P = 0.090$; Fig. 4, A and C). To test whether the hair bulge stem cells were able to mobilize (exit their quiescence state and migrate) out of the niche, we studied the response of G3 *Terc*$^{-/-}$ and G3 *Terc*$^{+/-*}$ LRCs to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment, a potent tumor promoter that activates LRCs to give numerous progeny (Flores et al., 2005).

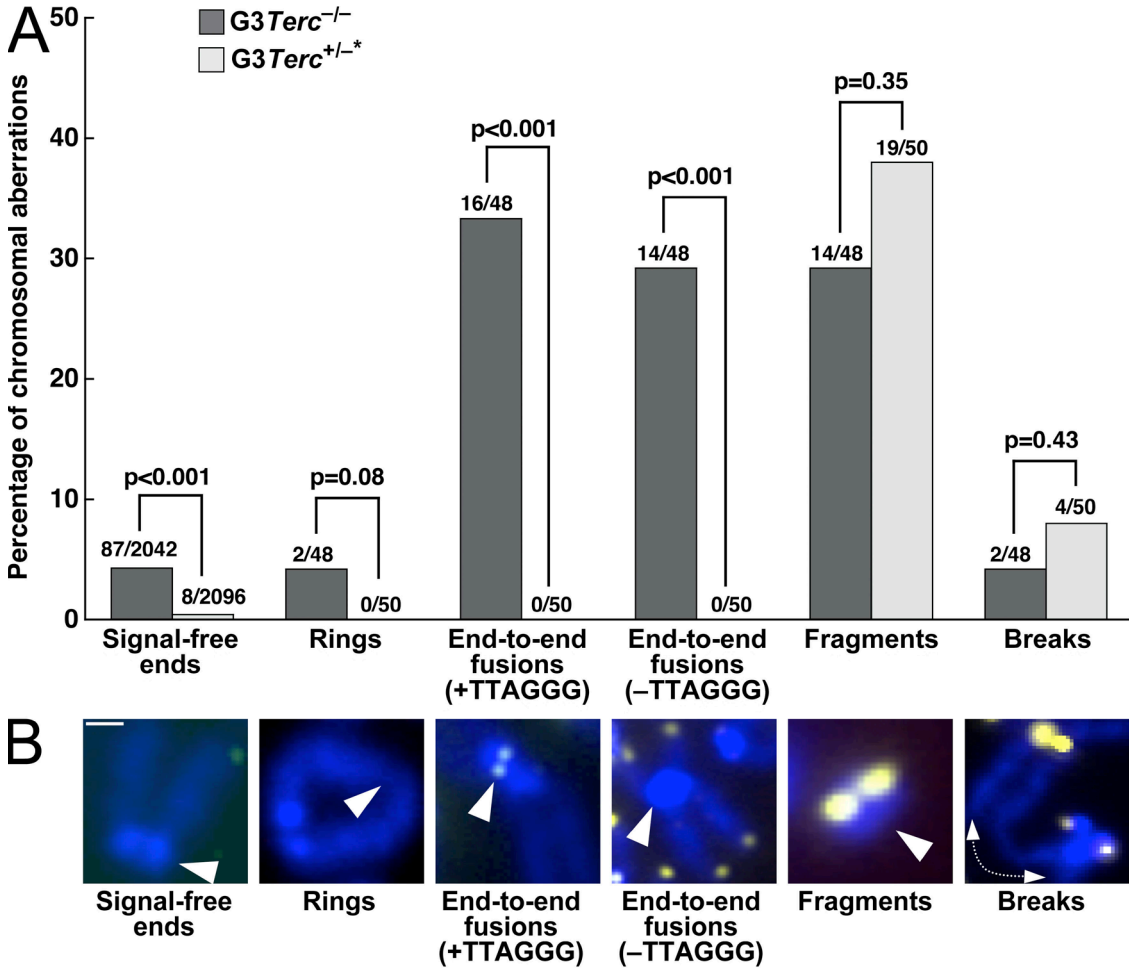
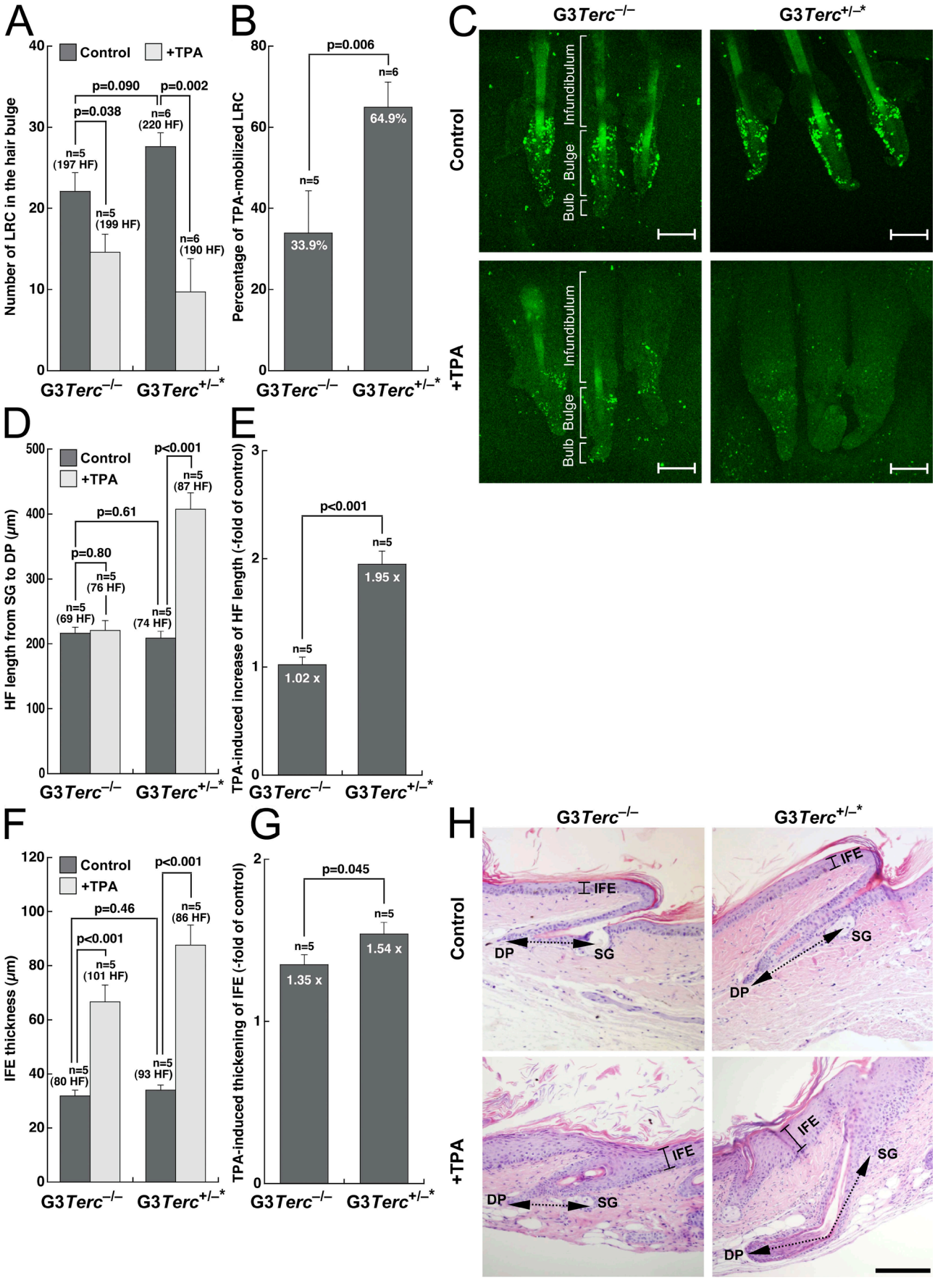


Figure 3. **Significant rescue of signal-free ends and end-to-end chromosomal fusions in G3 *Terc*$^{+/-*}$ keratinocytes.** (A) Quantification of the frequency of the indicated chromosomal aberrations in primary keratinocytes from G3 *Terc*$^{-/-}$ and G3 *Terc*$^{+/-*}$ mice. The total number of chromosomes scored for the analysis is indicated on top of each bar. Statistical comparisons using the χ^2 test are shown. (B) Representative examples of the indicated chromosomal aberrations are shown below the graph. Note that signal-free ends and end-to-end fusions are significantly rescued in Terc-reconstituted G3 mice compared with the G3 Terc-deficient littermates, which is in agreement with the fact that telomerase elongates short telomeres, thus preventing telomere dysfunction. Bar, 0.4 μm.

TPA treatment results in rapid disappearance of LRCs (Braun et al., 2003), skin hyperplasia (Gonzalez-Suarez et al., 2000), and entry of HFs into their anagen (growing) phase (Wilson et al., 1994). After TPA treatment, only 34% of the LRCs mobilized out of the HF niche in G3 *Terc*$^{-/-}$ mice (Fig. 4, B and C) compared with the previously reported 70% mobilization in wild-type mice of different genetic backgrounds (Flores et al., 2005), thus confirming the defective stem cell mobilization associated with short telomeres. In contrast, 65% of LRCs mobilized in TPA-treated G3 *Terc*$^{+/-*}$ mice ($P = 0.006$; Fig. 4 B). A similar rescue in HF stem cell mobilization defects was obtained when comparing G4 *Terc*$^{+/-*}$ mice to the corresponding G4 *Terc*$^{-/-}$ littermates (Fig. S1, A–C, available at http://www.jcb.org/cgi/content/full/jcb.200704141/DC1). Collectively, these results demonstrate that telomerase reintroduction in late generation G3 and G4 *Terc*$^{+/-*}$ mice significantly rescues epidermal HF

Figure 4. **Rescue of HF stem cell mobilization defects, as well as of skin and hair growth defects in late generation telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice upon TPA treatment.** (A and B) To assay mobilization of LRCs, mice of the indicated genotype were injected with BrdU and after 69 d, whole mounts of tail epidermis were collected from untreated and TPA-treated mice and stained with a BrdU antibody. The number of LRCs at the hair bulge in resting conditions or after stimulation with TPA in littermate G3 *Terc*$^{+/-*}$ and G3 *Terc*$^{-/-}$ mice are indicated (A). LRC numbers were calculated in at least five mice of each genotype (*n*), quantifying at least 197 follicles in the control group and at least 190 follicles in the TPA-treated group (A). Note a higher percentage of mobilization of LRCs in TPA-treated G3 *Terc*$^{+/-*}$ mice compared with G3 *Terc*$^{-/-}$ mice (B). (C) Representative confocal micrographs of tail follicles from G3 *Terc*$^{-/-}$ and G3 *Terc*$^{+/-*}$ mice were stained for BrdU (green) before (controls) and after (+TPA) TPA treatment. LRCs of all genotypes accumulate in the bulge (Bu) region of the HF. Bar, 80 μm. Note a greater disappearance of LRCs in *Terc*$^{+/-*}$ TPA-treated HFs compared with the G3 *Terc*$^{-/-}$ littermates. The different compartments (infundibulum, hair bulge, and hair bulb) of the HF are indicated. (D–G) Quantification of HF length from sebaceous glands (SG) to dermal papilla (DP; D and E) and of IFE thickness (F and G) in tail skin from mice of the indicated genotype. Histomorphometry was performed in five mice of each genotype (*n*), quantifying at least 69 follicles in the control group and at least 76 follicles in the TPA-treated group. Note the increased HF length and IFE thickness in TPA-treated G3 *Terc*$^{+/-*}$ mice compared with G3 *Terc*$^{-/-}$ littermates ($P < 0.05$ for both; E and G). (H) Representative tail skin sections from mice of the indicated genotypes before (control) and after (+TPA) TPA treatment. Bracketed continuous black lines mark IFE. Black dashed double-pointed arrows mark HF length from sebaceous glands to dermal papilla. Bar, 150 μm. Error bars in A, B, and D–G represent SEM.



stem cell mobilization defects compared with the G3 and G4 *Terc*$^{-/-}$ littermates.

In agreement with the defects in HF stem cell mobilization associated with short telomeres, HF length was not significantly increased in G3 *Terc*$^{-/-}$ mice after TPA treatment ($P = 0.80$; Fig. 4, D, E, and H), reflecting a defective HF anagen response in these mice after TPA treatment (Flores et al., 2005). Again, telomerase reintroduction rescued this defect in G3 *Terc*$^{+/-*}$ littermates, where HF length was significantly increased in response to TPA treatment compared with resting nontreated skin ($P < 0.001$; Fig. 4, D, E, and H), suggesting that telomerase is sufficient to restitute skin homeostasis in these mice. Similar results were obtained for increased interfollicular epidermis (IFE) thickness in response to TPA treatment. Again, telomerase reintroduction in G3 *Terc*$^{+/-*}$ littermates resulted in increased IFE hyperplasia compared with littermate G3 *Terc*$^{-/-}$ mice in response to TPA treatment ($P < 0.05$; Fig. 4, F–H). To study whether the increased IFE hyperplasia in G3 *Terc*$^{+/-*}$ mice compared with the G3 *Terc*$^{-/-}$ controls was associated with significant differences in cell proliferation or apoptosis, we performed immunohistochemistry of skin sections with antibodies against Ki67 and caspase 3 to detect proliferating and apoptotic cells, respectively (Materials and methods). As shown in Fig. S2 (available at http://www.jcb.org/cgi/content/full/jcb.200704141/DC1), we did not detect considerable differences in the percentage of Ki67-positive cells between G3 *Terc*$^{+/-*}$ and G3 *Terc*$^{-/-}$ either in resting skin conditions or upon TPA treatment. Similarly, we were unable to detect caspase 3–positive cells in the skin of G3 *Terc*$^{+/-*}$ and G3 *Terc*$^{-/-}$ mice, suggesting that apoptosis is not a major cellular response to critically short telomeres in the skin (Fig. S3). Finally, we studied whether there were differences in skin differentiation markers between G3 *Terc*$^{+/-*}$ and G3 *Terc*$^{-/-}$ mice by performing immunohistochemistry with antibodies against K14 and p63, two skin basal-layer markers whose expression is normally reduced at the suprabasal skin layers (Materials and methods). We could not detect significant differences in the percentage of cells or in the number of keratinocyte layers positive for these markers (Figs. S4 and S5).

Next, we used hair-plucking experiments as an independent way to induce entry of HFs into their anagen phase (Flores et al., 2005; Materials and methods). In control resting skin conditions, we did not detect differences in back skin HF length and dermis thickness between G3 *Terc*$^{-/-}$ and G3 *Terc*$^{+/-*}$ littermates ($P = 0.71$ and $P = 0.49$, respectively; Fig. 5, A, C, and E). Upon hair plucking, however, G3 *Terc*$^{+/-*}$ mice showed a significantly increased back skin HF length and dermis thickness compared with the corresponding G3 *Terc*$^{-/-}$ littermates ($P = 0.04$ and $P = 0.09$, respectively; Fig. 5, A–E), again demonstrating that telomerase reintroduction in mice with short telomeres is able to improve the ability of epidermal HF stem cells to mobilize and regenerate the hair and skin.

Rescue of proliferative potential of epidermal stem cells ex vivo in late generation telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice

It has been previously described that the epidermal stem cell defects observed in late generation telomerase-deficient mice are cell autonomous, as indicated by a defective clonogenic potential of these cells ex vivo (Flores et al., 2006). Individual colonies in clonogenic assays have been proposed to derive from single stem cells (Barrandon and Green, 1987; Materials and methods). Here, we performed clonogenic assays to compare the proliferation potential of telomerase-deficient G3 *Terc*$^{-/-}$ and telomerase-reconstituted G3 *Terc*$^{+/-*}$ epidermal stem cells (Materials and methods). In agreement with the in vivo results shown in Fig. 4, primary keratinocytes from newborn G3 *Terc*$^{-/-}$ mice formed fewer and smaller colonies than those from wild-type controls ($P < 0.001$; Fig. 6, A and B), reflecting the previously described defective capacity of late generation *Terc*-null epidermal stem cells to proliferate ex vivo (Flores et al., 2005). Interestingly, the defective clonogenic potential of these cells was significantly corrected in telomerase-reconstituted G3 *Terc*$^{+/-*}$ keratinocytes compared with the corresponding G3 *Terc*$^{-/-}$ controls ($P = 0.006$; Fig. 6, A and B), demonstrating that telomerase reintroduction ameliorates the ex vivo proliferative capacity of epidermal stem cells from late generation telomerase-deficient mice.

Reintroduction of the telomerase Terc gene in G3 Terc-deficient mice is sufficient to rescue their small body-size phenotype

We have previously described that late generation telomerase-deficient mice show a small body-size phenotype at the time of birth, which is associated with shorter telomeres in these mice as well as with a decreased clonogenic potential of epidermal stem cells in vitro (Flores et al., 2005). These observations opened the intriguing possibility that body size and stem cell proliferative potential may be mechanistically related. Here, we first confirmed that newborn G3 *Terc*$^{-/-}$ mice showed a significantly lower body weight at the time of birth compared with the wild-type controls ($P < 0.001$; Fig. 6, C and D, left), which again was concomitant with a lower clonogenic potential of keratinocytes derived from G3 *Terc*$^{-/-}$ mice compared with the wild types (Fig. 6, A and B). Interestingly, the small body-size phenotype of newborn G3 *Terc*$^{-/-}$ mice was significantly corrected by telomerase reintroduction into G3 *Terc*$^{+/-*}$ newborns, paralleling the rescue of stem cell phenotypes in these mice (Figs. 4–6). This significant rescue of the small body-size phenotype observed in newborns was maintained when comparing age-matched adult (2 mo old) G3 *Terc*$^{+/-*}$ mice to the corresponding G3 *Terc*$^{-/-}$ littermates ($P < 0.001$; Fig. 6, C and D, right). These results support that the small body size of telomerase-deficient mice may be linked to decreased stem cell functionality.

Reintroduction of the telomerase Terc gene in G3 Terc-deficient mice is sufficient to rescue a normal life span in the absence of increased cancer

The results presented here for epidermal HF stem cells open the possibility that telomerase reintroduction into mice with critically short telomeres may be sufficient to restore stem cell functionality in different tissues, thus rescuing life span and long-term survival. To address this, we studied the long-term survival and maximum life span of both telomerase-reconstituted G4 *Terc*$^{+/-*}$ and telomerase-deficient G4 *Terc*$^{-/-}$ littermates,

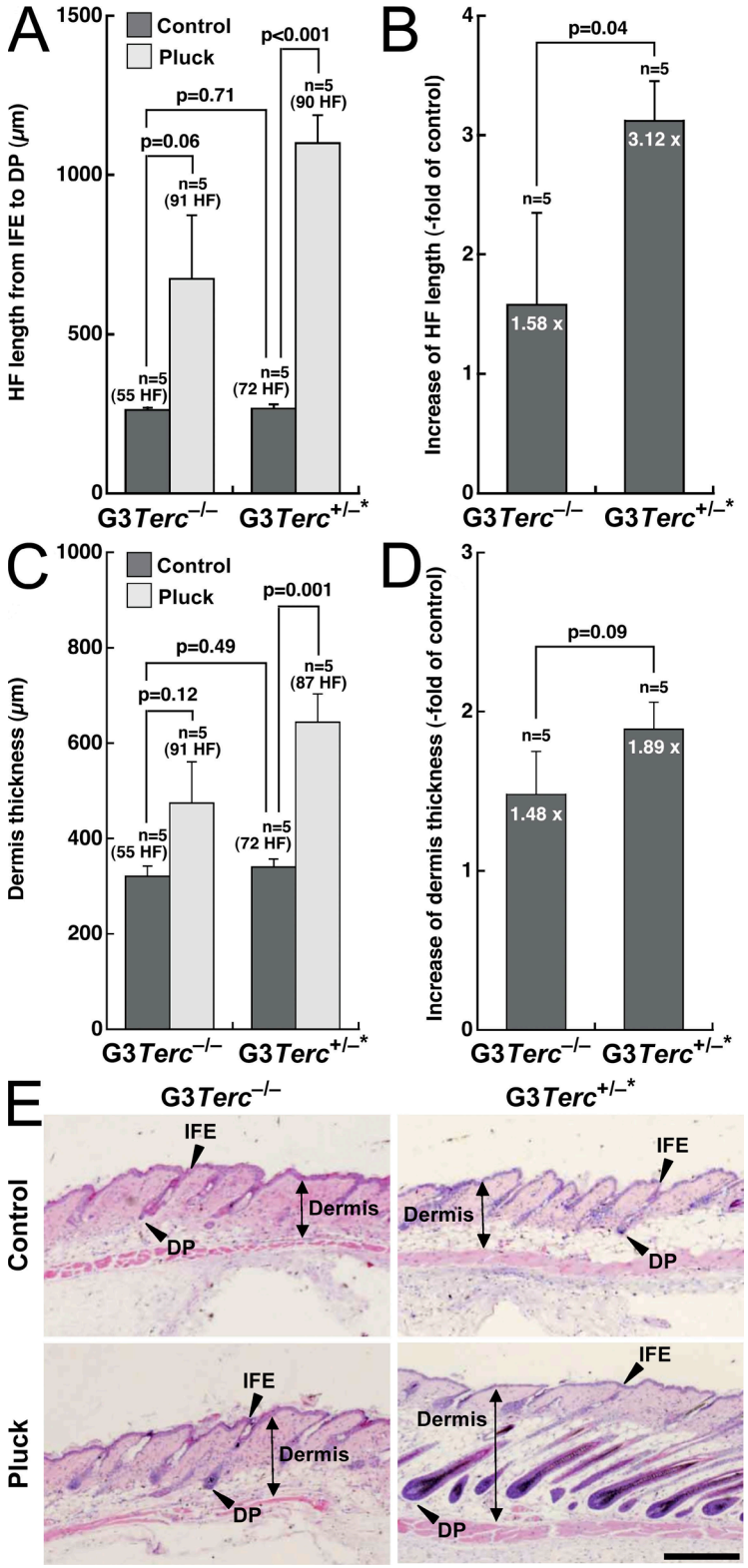


Figure 5. **Rescue of skin and hair growth defects in late generation telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice upon hair plucking of back skin.** (A–D) Quantification of HF length from IFE to dermal papilla (A and B) and of dermis thickness (C and D) in back skin from mice of the indicated genotype. Histomorphometry was performed in five mice of each genotype, quantifying at least 55 follicles in the control groups and at least 87 follicles in the TPA-treated groups. Note the increased HF length and dermis thickness in G3 *Terc*$^{+/-*}$ mice compared with G3 *Terc*$^{-/-}$ littermates after plucking (B and D). Error bars represent SEM. (E) Representative back skin sections from mice of the indicated genotypes before (control) and after (pluck) plucking. Black double-pointed arrows mark dermis thickness. Arrowheads mark HF length from IFE to dermal papilla. Bar, 480 μm.

compared with that of control wild-type mice as well as control nonreconstituted heterozygous *Terc*$^{+/-}$ mice (Fig. 7, A–C). First, we confirmed a dramatic decrease in the maximum life span of G4 *Terc*$^{-/-}$ mice compared with wild-type and *Terc*$^{+/-}$ controls, which went from ~130 and 140 wk, respectively, to <50 wk in the case of G4 *Terc*$^{-/-}$ mice ($P < 0.001$; Fig. 7 A). G4 *Terc*$^{-/-}$ mice also showed a significantly decreased median survival compared with the wild-type and *Terc*$^{+/-}$ controls ($P < 0.001$; Fig. 7 B). Importantly, telomerase-reconstituted G4 *Terc*$^{+/-*}$ mice showed a survival curve and a median survival that is indistinguishable from that of wild-type and *Terc*$^{+/-}$ controls (Fig. 7, A and B, NS; $P = .0.56$), indicating

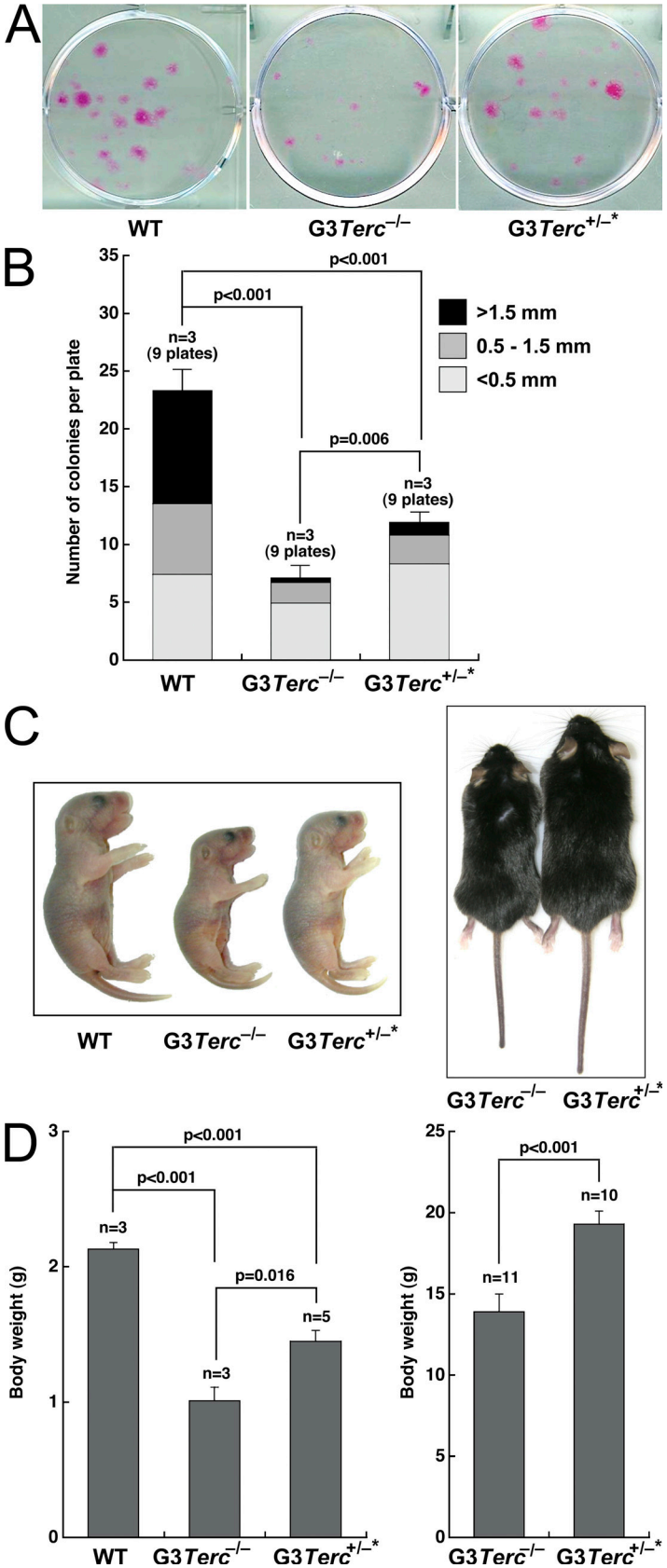


Figure 6. **Rescue of proliferative potential of epidermal stem cells ex vivo and of small body-size phenotype in late generation telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice.** (A) Representative images of number and size of macroscopic colonies obtained from isolated keratinocytes of the indicated genotypes. Note more abundant and larger colonies in G3 *Terc*$^{+/-*}$ mice compared with G3 *Terc*$^{-/-}$ littermates. (B) Quantification of size and number of macroscopic colonies obtained from isolated keratinocytes of the indicated genotype purified from 2-d-old mice and cultured for 10 d on J2-3T3 mitomycin C–treated feeder fibroblasts. (C) Representative images of newborn (left) and 2-mo-old (right) mice of the indicated genotype. Note a small body size in G3 *Terc*$^{-/-}$ mice compared with wild-type controls and that this small body-size phenotype is largely rescued in telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice. (D) Quantification of body weight in newborn (left) and 2-mo-old (right) mice of the indicated genotypes. Note significantly reduced body size in newborn G3 *Terc*$^{-/-}$ mice compared with both wild-type controls and telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice ($P < 0.001$ for both) and the significantly reduced body size in adult G3 *Terc*$^{-/-}$ mice compared with telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice ($P < 0.001$). Error bars in B and D represent SEM.

that telomerase reintroduction into mice with critically short telomeres is sufficient to restore a normal long- term survival in these mice.

Next, we studied whether telomerase-reconstituted G4 *Terc*$^{+/-*}$ mice had a different spectrum of cancer and aging pathologies compared with control wild-type and *Terc*$^{+/-}$

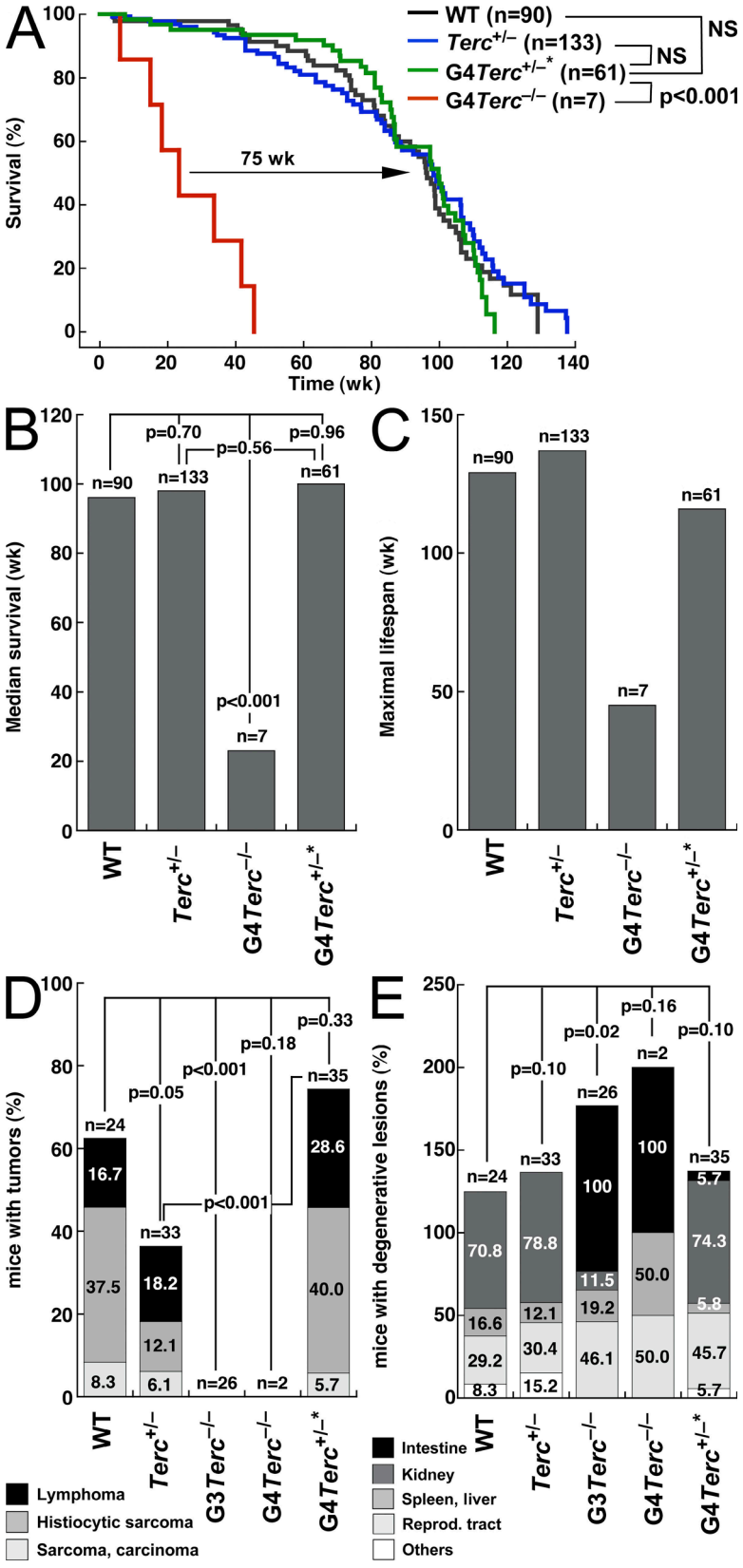
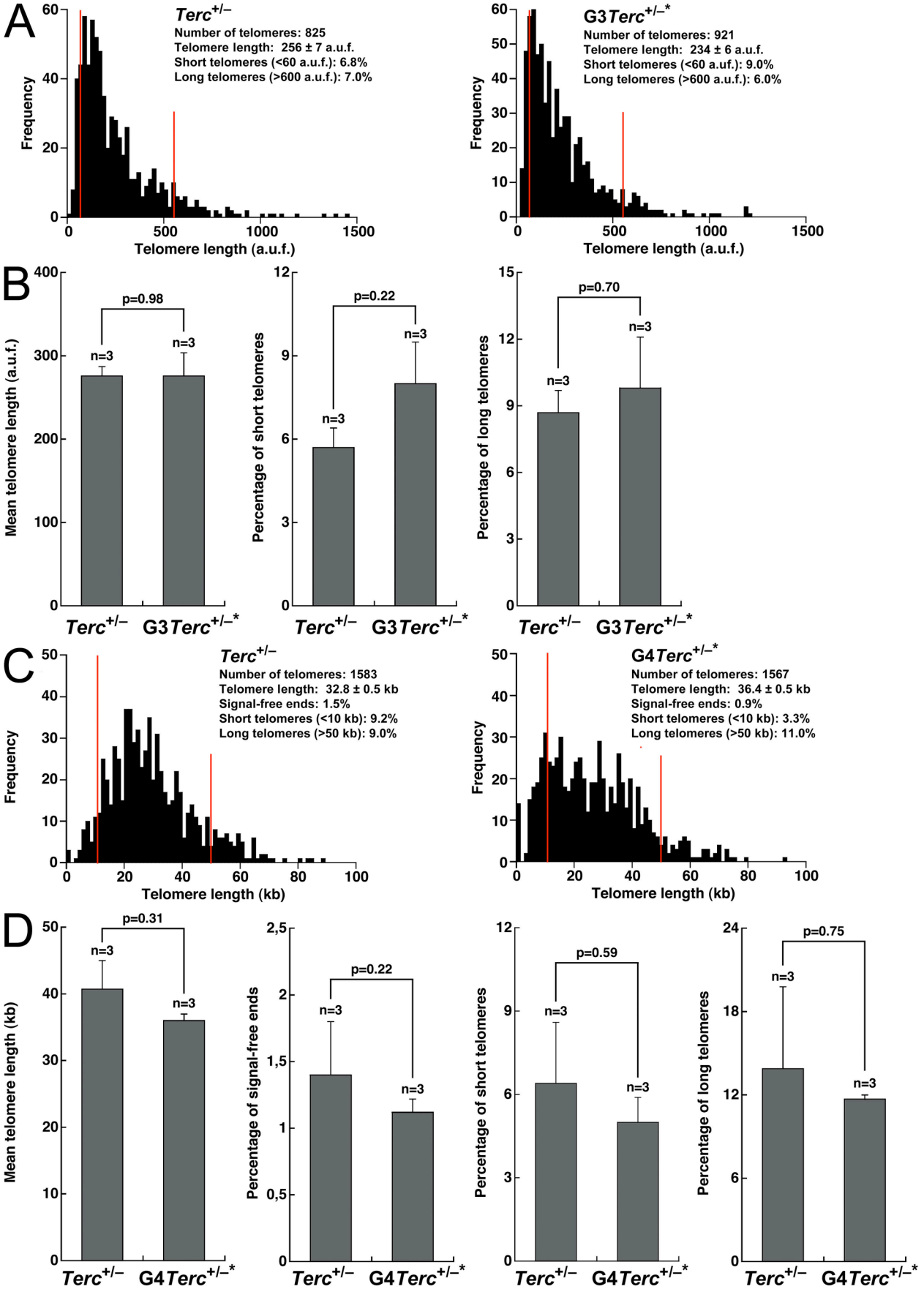


Figure 7. **Rescue of organismal life span in late generation telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice.** (A) Kaplan-Meyer survival curve of mice of the indicated genotype. *n* is the number of mice of each genotype. Statistical comparisons using the log rank test are also shown. Note the significant rescue in survival of telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice compared with G3 *Terc*$^{-/-}$ mice ($P < 0.001$). (B) Median survival obtained from Kaplan-Meyer plots of mice of the indicated genotypes. Statistical comparisons using the log rank test are also shown. Note the significantly reduced median survival of G3 *Terc*$^{-/-}$ mice compared with the other genotypes ($P < 0.001$). (C) Maximal lifespan of mice of the indicated genotypes. Note the decreased maximal life span of G3 *Terc*$^{-/-}$ mice and how this is corrected in telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice. (D) Percentage of mice of the indicated genotype showing malignant tumors (lymphoma, histiocytic sarcoma, or sarcoma/carcinoma) at the time of death. Statistical comparisons using the χ^2 test are also shown. Note the decreased incidence of malignant tumors in G3 and G4 *Terc*$^{-/-}$ mice in agreement with a tumor-suppressor role associated with telomerase deficiency and short telomeres. Telomerase-reconstituted G4 *Terc*$^{+/-*}$ mice show a similar tumor incidence to that of wild-type controls. (E) Percentage of mice of the indicated genotype showing degenerative pathologies (atrophies) in the indicated tissues at the time of death. Statistical comparisons using the χ^2 test are also shown. Note the decreased incidence of intestinal atrophies in telomerase-reconstituted G4 *Terc*$^{+/-*}$ mice compared with G3 and G4 *Terc*$^{-/-}$ mice.

heterozygous mice. Telomerase-deficient G3 and G4 *Terc*$^{-/-}$ mice have a dramatic reduction in the incidence of malignant tumors compared with wild-type and normal *Terc*$^{+/-}$ heterozygous mice (Fig. 7 D), which is in agreement with a potent tumor suppressor role for short telomeres in the context of telomerase deficiency (Greenberg et al., 1999; Gonzalez-Suarez et al., 2000; Blasco and Hahn, 2003). In contrast, G4 *Terc*$^{+/-*}$ mice showed a similar or higher incidence of malignant tumors at time of



death (lymphomas, sarcomas, and carcinomas) compared with that of wild-type and *Terc*$^{+/-}$ heterozygous controls, respectively (Fig. 7 D), suggesting that telomerase reintroduction is sufficient to sustain normal tumorigenesis in G4 *Terc*$^{+/-*}$ mice. Of notice, the increased tumorigenesis in G4 *Terc*$^{+/-*}$ mice compared with nonreconstituted *Terc*$^{+/-}$ heterozygous controls ($P < 0.001$; Fig. 7 D) may be related to the fact that not all chromosomal defects associated with short telomeres are rescued by telomerase reintroduction (i.e., fragments and breaks; Fig. 3).

Finally, telomerase-reconstituted G4 *Terc*$^{+/-*}$ mice also showed a significant rescue of degenerative pathologies compared with G3 and G4 *Terc*$^{-/-}$ mice. In particular, atrophies of the small intestine appeared in only 6% of G4 *Terc*$^{+/-*}$ mice at time of death compared with 100% of the G3 and G4 *Terc*$^{-/-}$ mice (Fig. 7 E). Importantly, degenerative pathologies in G4 *Terc*$^{+/-*}$ mice showed a similar incidence to those of aged-matched normal *Terc*$^{+/-}$ controls with a similar dose of the Terc allele, illustrating a complete rescue of degenerative pathologies associated with late generation *Terc*$^{-/-}$ mice. Collectively, these results suggest that telomerase reconstitution into mice with critically short telomeres is sufficient to confer a normal life span and normal aging in the absence of abnormally increased tumorigenesis.

Finally, in agreement with the similar incidence of degenerative pathologies and cancer, mean telomere length, as well as the percentages of short and long telomeres, was indistinguishable between telomerase-reconstituted G3/G4 *Terc*$^{+/-*}$ mice and the *Terc*$^{+/-}$ controls in both age-matched adult skin keratinocytes (10 mo old; Fig. 8, A and B) and primary splenocytes (12–24 mo old; Fig. 8, C and D), suggesting that telomerase activity with a normal telomere length is able to provide homeostasis during the life span of these mice.

Discussion

The mechanisms by which short telomeres negatively impact on organismal aging and life span are still far from being understood. One of the proposed mechanisms, which has gained increasing experimental support, is the progressive loss of stem cell functionality associated with critically short telomeres. Evidence for this comes from the study of the telomerase-deficient mouse model, which shows impaired stem cell functionality in several tissues including the bone marrow, the brain, and the skin (Lee et al., 1998; Samper et al., 2002; Ferron et al., 2004; Flores et al., 2005). In particular, we have recently shown that late generation telomerase-deficient mice show an impaired ability of epidermal stem cells to mobilize out of their niches and to regenerate the skin and the hair. This defective stem cell behavior anticipates the fact that telomerase-deficient mice show premature aging of the hair and the skin as well as an increased resistance to developing skin cancer (Lee et al., 1998; Herrera et al., 1999; Rudolph et al., 1999; Gonzalez-Suarez et al., 2000), supporting the notion that short telomeres provoke aging by impairing the functionality of stem cells.

Here, we provide further support for a stem cell theory of telomere-mediated aging by showing that telomerase reintroduction in late generation telomerase-deficient mice is sufficient to restore a normal behavior of epidermal HF stem cells and a normal skin functionality in these mice, therefore supporting the notion that stem cells are important players in the known role of telomeres and telomerase in aging. In this regard, we show that epidermal HF stem cell defects of late-generation Terc-deficient mice are independent of proliferation rates, apoptosis, or expression of the K14 and p63 differentiation markers in the skin, similar to *Terc*$^{-/-}$ and *Terc*$^{+/-*}$ mice. Indeed, the defective mobilization ability of epidermal HF stem cells anticipates the premature skin aging phenotypes of these Terc-deficient mice. Furthermore, we demonstrate that telomerase reconstitution in the context of very short telomeres not only corrects epidermal HF stem cell defects in newborn mice but is also sufficient to sustain a long-term normal organismal life span in these mice by preventing organismal aging in the absence of increased cancer. It is important to highlight that telomerase-reconstituted mice show a telomere length that is indistinguishable from that of normal, nonreconstituted, Terc heterozygous mice, indicating that telomerase activity not only elongates short telomeres but is able to restitute a normal telomere-length homeostasis during the life span of these mice.

Finally, these observations support the idea that therapies based on telomerase activation may be effective in correcting the proaging effects of short telomeres in the absence of increased risk of carcinogenesis. This is of particular relevance in the case of premature aging diseases characterized by decreased levels of telomerase activity and shorter telomeres, such as some cases of dyskeratosis congenita and aplastic anemia, which result in premature death associated with a defective tissue renewal capacity (bone marrow and skin) and increased cancer (Mason et al., 2005).

Figure 8. **Pronounced rescue of short telomeres in Terc-reconstituted and Terc-deficient keratinocytes and splenocytes.** (A) Telomere length histograms obtained by Q-FISH on tail skin sections. The histogram shown is representative of three independent age-matched (10 mo old) pairs of *Terc*$^{+/-}$ and telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice (see B). Red lines facilitate visualization of short (<60 a.u.f.) and long (>600 a.u.f.) telomeres. The total number of telomere dots used for the quantification and the percentage of short and long telomeres are indicated. Note very similar telomere length distributions in age-matched *Terc*$^{+/-}$ and telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice. (B) Mean telomere length is indistinguishable between age-matched *Terc*$^{+/-}$ and telomerase-reconstituted G3 *Terc*$^{+/-*}$ mice ($P = 0.98$). Concomitantly, these mice show similar percentages of short telomeres ($P = 0.22$) and long telomeres ($P = 0.70$). Data are mean values ± SEM for three independent pairs of G3 *Terc*$^{-/-}$ and G3 *Terc*$^{+/-*}$ mice. (C) Telomere length histograms obtained by Q-FISH directly on metaphases of freshly isolated splenocytes. The histogram shown is representative of three age-matched (1–2 yr old) pairs of *Terc*$^{+/-}$ and telomerase-reconstituted G4 *Terc*$^{+/-*}$ mice. In this case, arbitrary units of fluorescence were converted into kilobases as described in Materials and methods. Red lines facilitate visualization of short (<10 Kb) and long (>50 Kb) telomeres. The total number of individual telomeres used for the quantification and the percentage of short and long telomeres are indicated. Note very similar telomere length distributions in age-matched *Terc*$^{+/-}$ and telomerase-reconstituted G4 *Terc*$^{+/-*}$ mice. (D) Mean telomere length is indistinguishable between age-matched *Terc*$^{+/-}$ and telomerase-reconstituted G4 *Terc*$^{+/-*}$ mice ($P = 0.31$). Concomitantly, these mice show similar percentages of signal-free ends ($P = 0.22$), short telomeres (<10 Kb; $P = 0.59$), and long telomeres (>50 Kb; $P = 0.75$). Data are mean values ± SEM for three independent *Terc*$^{+/-}$ and three independent G4 *Terc*$^{+/-*}$ mice.

Materials and methods

Generation and genotyping of mice
To generate littermate G3/G4 *Terc*$^{+/-*}$ and G3/G4 *Terc*$^{-/-}$ mice, G2/G3 *Terc*$^{-/-}$ males were crossed with *Terc*$^{+/-}$ females (Blasco et al., 1997). Genotyping was performed as described in Blasco et al. (1997). Note that littermate G3/G4 *Terc*$^{+/-*}$ and G3/G4 *Terc*$^{-/-}$ mice are of an exact genetic background (C57BL6) as if they were derived from the same parents.

Mouse handling
Mouse colonies were generated in a pure C57BL6 background and maintained at the Spanish National Cancer Center under specific pathogen-free conditions in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations.

Telomeric repeat amplification protocol
Primary mouse embryonic fibroblasts (MEFs) were trypsinized and washed in PBS, and S-100 extracts were prepared as described in Blasco et al. (1997). Three protein concentrations were used for each sample (5, 2, and 1 μg). Extension and amplification reactions and electrophoresis were performed as described in Blasco et al. (1997). A negative control was included by preincubating each MEF extract with RNase for 10 min at 30°C before the extension reaction. An internal control for PCR efficiency was included (TRAPeze kit; Oncor).

Treatment regimens
To induce LRC mobilization, IFE hyperplasia, and anagen entry, tail skin from 71-d-old mice in the telogen (resting) phase of the hair cycle was topically treated every 48 h with TPA (20 nM in acetone) for a total of four doses. The control mice were treated with acetone only. 24 h after the last TPA treatment, mice were killed and the tail skin was analyzed. To induce anagen by physical stimulation, dorsal HFs in the telogen phase of the hair cycle were plucked from the back skin of 60-d-old G3 *Terc*$^{+/-*}$ mice and the corresponding G3 *Terc*$^{-/-}$ controls. 10 d after plucking, dorsal skins were harvested and prepared for histology.

Labeling of LRCs
LRCs were obtained as described in Bickenbach et al. (1986), Cotsarelis et al. (1990), and Braun et al. (2003), with some modifications. In brief, litters of neonatal mice were injected with 50 mg/kg of bodyweight BrdU (Sigma-Aldrich) diluted in PBS. Each animal received a daily injection beginning at day 4 of life for a total of 5 d. After the labeling period, mice were allowed to grow for 60 d before the initiation of any treatment. Cells retaining the label at the end of the treatment were identified as LRCs.

Preparation of whole mounts
Whole mounts of mouse tail epidermis were prepared as previously described in Braun et al. (2003). In brief, after mice were killed with CO_2 and their tails were amputated, skin was peeled from the tails and incubated in 5 mM EDTA in PBS at 37°C for 4 h. Using forceps, intact sheets of epidermis were separated from the dermis and fixed in neutral-buffered formalin for 2 h at room temperature. Fixed epidermal sheets were maintained in PBS containing 0.2% sodium azide at 4°C before labeling.

Immunofluorescence of epidermal sheets
To detect LRCs in whole mounts of the tail skin, fixed epidermal sheets were blocked and permeabilized by incubation for 30 min in a modified phosphate buffer (Braun et al., 2003) containing 0.5% BSA and 0.5% Triton X-100 in TBS. Subsequently, epidermal sheets were immersed for 30 min in 2 M HCl at 37°C, incubated overnight with a mouse anti-BrdU antibody conjugated with fluorescein (Roche) at 1:50 in modified PB buffer, washed four times in PBS containing 0.2% Tween 20, and mounted in Vectashield (Vector Laboratories).

Confocal microscopy
A laser scanning confocal microscope (TCS-SP2-AOBS; Leica) was used to obtain fluorescence images. Image stacks of 60–80 μm were obtained through the z dimension at steps 1.0 μm apart, using a PL APO 20×/0.70 PH2 (Leica) as lens. Maximum intensity projections of the image stacks were then generated using LCS Software (Leica).

Pathology analyses
Mice were killed when they showed signs of poor health, such as reduced activity or weight loss, and subjected to exhaustive histopathological analysis. The organs we analyzed for age-related degenerative pathologies were the intestine (atrophy of the small and large intestine), kidney (glomerulonephritis and tubular degeneration), spleen (atrophy, hemosiderosis, and myeloid and lymphoid hyperplasia), liver (congestion, vacuolar degeneration, microgranuloma, and steatosis), testis (atrophy and ectasis of seminal vesicles), ovary (atrophy), uterus (cystic endometrial hyperplasia), skin (benign hyperplasia), lung (congestion), heart (congestion and cardiomyopathy), and brain (calcification).

Histology and immunohistochemistry of skin
Tail or back skin samples were harvested from mice and fixed overnight in neutral-buffered formalin at 4°C, dehydrated through graded alcohols and xylene, and embedded in paraffin. For determination of IFE thickness, dermis thickness, and HF length, dissected skin was cut parallel to the spine and sections were cut perpendicular to the skin surface to obtain longitudinal HF sections. 5-μM sections were used for hematoxylin-eosin staining.

For immunohistochemistry, tail skin samples were sectioned at 2–3 μm and processed with 10 mM sodium citrate, pH 6.5, cooked under pressure for 2 min. Slides were washed in water, and then in TBS Tween 20 0.5%, blocked with peroxidase, washed with TBS Tween 20 0.5% again, and blocked with FBS followed by another wash. The slides were incubated with the primary antibodies: rabbit monoclonal to Ki-67 antibody (prediluted; SP6; Master Diagnostica), rabbit polyclonal active caspase 3 at 1:200 (R&D Systems), mouse monoclonal p63 at 1:100 (clone A48, Neomarker), or rabbit polyclonal K14 at 1:100 (Neomarker). Slides were then incubated with secondary antibodies conjugated with peroxidase (DakoCytomation), goat anti-rabbit (1:50) in the case of Ki-67, active caspase 3, K14, and mouse on mouse (Vector Laboratories) in the case of p63. For signal development, DAB (DakoCytomation) was used as a substrate. Sections were lightly counterstained with hematoxylin and analyzed by light microscopy.

Isolation of newborn keratinocytes
2-d-old mice were killed and soaked in betadine (5 min), in a PBS antibiotic solution (5 min), in 70% ethanol (5 min), and again in a PBS antibiotic solution (5 min). Limbs and tail were amputated and the skin was peeled off using forceps. Skins were then soaked in PBS (2 min), PBS antibiotic solution (2 min), 70% ethanol (1 min), and again in PBS antibiotic solution (2 min). Using forceps, each skin was floated on the surface of 1× trypsin solution (4 ml on a 60-mm cell culture plate; Sigma-Aldrich) for 16 h at 4°C. Skins were transferred to a sterile surface. The epidermis was separated from the dermis using forceps, minced, and stirred at 37°C for 30 min in serum-free Cnt-02 medium (CELLnTEC Advanced Cell Systems AG). The cell suspension was filtered through a sterile Teflon mesh (Cell Strainer 0.7 m; BD Biosciences) to remove cornified sheets. Keratinocytes were then collected by centrifugation (160 *g*) for 10 min and seeded on collagen I–precoated cell culture plates (BD Biosciences).

Colony-forming assay and culture conditions
1,000 mouse keratinocytes per genotype were seeded onto 10 μg/ml mitomycin C (2 h), treated with J2-3T3 fibroblasts (10^5 per well, 6-well dishes), and grown at 37°C/5% CO_2 in Cnt-02 medium. After 10 d of cultivation, dishes were rinsed twice with PBS, fixed in 10% formaldehyde, and then stained with 1% Rhodamine B to visualize colony formation. Colony size and number were measured using three dishes per experiment.

Telomere length analysis by Q-FISH
Freshly isolated splenocytes were obtained by squeezing the spleen through a cell strainer (70 μm; Nylon; BD Biosciences). Red cells were lysed by osmotic shock, and the splenocytes were resuspended in RPMI 1640 containing 10% FBS and 0.55 μM β-mercaptoethanol. Concanavalin A (Sigma-Aldrich) was added to a concentration of 5 μg/ml and splenocytes were grown for 48 h. The cells were incubated with 0.1 μg/ml colcemide (Invitrogen) for 2 h and fixed in methanol/acetic acid (3:1). Q-FISH was performed as described in Herrera et al. (1999) and Samper et al. (2000). To correct for lamp intensity and alignment, images from FluoroSpheres (fluorescent beads; Invitrogen) were analyzed using the TFL-Telo software (provided by P. Lansdorp, Terry Fox Laboratory, Vancouver, Canada). Telomere fluorescence values were extrapolated from the telomere fluorescence of lymphoma cell lines LY-R (R cells) and LY-S (S cells) with known telomere lengths of 80 and 10 kb, respectively. There was a linear correlation (r^2 = 0.999) between the fluorescence intensity of the R and S telomeres. We recorded the images using a camera (CCK; COHU) on a fluorescence microscope (DMRb; Leica). A mercury vapor lamp (CS 100 W-2; Philips) was used as a source. We captured the images using the Q-FISH software (Leica) in a linear acquisition mode to prevent oversaturation of fluorescence intensity.

We used the TFL-Telo software (Zijlmans et al., 1997) to quantify the fluorescence intensity of telomeres from at least 10 metaphases for each data point.

Exponentially growing primary keratinocytes were fixed in methanol/acetic acid, and Q-FISH of interphase nucleus was performed. For Q- FISH in tail skin, paraffin-embedded tail sections were deparaffinated. Both keratinocytes and deparaffinated sections of tail skin were hybridized with a PNA-telomeric probe and telomere fluorescence was determined as described in Gonzalez-Suarez et al. (2000) and Muñoz et al. (2005). More than 60 nuclei from each mouse and condition were captured at 100 magnification using a microscope (CTR MIC; Leica) and a camera (High Performance CCD; COHU). Telomere fluorescence was integrated using spot IOD analysis in the TFL-TELO program (Zijlmans et al., 1997).

Cytogenetic analysis using telomere Q-FISH on metaphases

Metaphases from keratinocytes of the indicated genotypes were obtained by adding 1 μg/ml colcemide (Invitrogen) to primary keratinocytes during 5 h and then fixing in methanol/acetic acid (3:1). Q-FISH was performed as described in Herrera et al. (1999) and Samper et al. (2000). For analysis of chromosomal aberrations, 50 metaphases per genotype were analyzed by superimposing the telomere image on the DAPI image using the TFL-telo software.

Statistical analysis

Unless otherwise indicated, data are given as mean values ± SEM of *n* and have been analyzed for statistically significant differences using *t* test.

Online supplemental material

Fig. S1 shows rescue of HF stem cell mobilization defects in late generation telomerase-reconstituted G4 *Terc*$^{+/-*}$ mice. Fig. S2 shows similar proliferation rates in G3 *Terc*$^{-/-}$ and G3 *Terc*$^{+/-*}$ tail skin. Fig. S3 shows no detectable apoptosis in the skin of G3 *Terc*$^{+/-*}$ mice and G3 *Terc*$^{-/-}$ siblings. Fig. S4 shows no differences in p63 expression between *Terc*$^{-/-}$ and G3 *Terc*$^{+/-*}$ tail skin. Fig. S5 shows no differences in keratin 14 expression between *Terc*$^{-/-}$ and G3 *Terc*$^{+/-*}$ tail skin. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200704141/DC1.

Thanks to E. Santos and R. Serrano for mouse care and genotyping.

I. Siegel-Cachedenier is a predoctoral fellow funded by the Spanish Ministry of Education and Culture. I. Flores is a Ramon y Cajal senior scientist. M.A. Blasco's laboratory is funded by the Ministry of Education and Science (SAF2001-1869, GEN2001-4856-C13-08), Comunidad Autonoma de Madrid (08.1/0054/01), European Union (TELOSENS FIGH-CT-2002-00217, INTACT LSHC-CT-2003-506803, ZINCAGE FOOD-CT-2003-506850, RISC-RAD FI6R-CT-2003-508842, MOL CANCER MED LSHC-CT-2004-502943), and Josef Steiner Award (2003).

Submitted: 4 January 2007
Accepted: 25 September 2007

References

Armanios, M.Y., J.J. Chen, J.D. Cogan, J.K. Alder, R.G. Ingersoll, C. Markin, W.E. Lawson, M. Xie, I. Vulto, J.A. Phillips III, et al. 2007. Telomerase mutations in families with idiopathic pulmonary fibrosis. *N. Engl. J. Med.* 356:1317–1326.

Barrandon, Y., and H. Green. 1987. Three clonal types of keratinocyte with different capacities for multiplication. *Proc. Natl. Acad. Sci. USA*. 84:2302–2306.

Bickenbach, J.R., J. McCutecheon, and I.C. Mackenzie. 1986. Rate of loss of tritiated thymidine label in basal cells in mouse epithelial tissues. *Cell Tissue Kinet.* 19:325–333.

Blackburn, E.H. 2001. Switching and signaling at the telomere. *Cell*. 106:661–673.

Blasco, M.A. 2005. Telomeres and human disease: ageing, cancer and beyond. *Nat. Rev. Genet.* 6:611–622.

Blasco, M.A., and W.C. Hahn. 2003. Evolving views of telomerase and cancer. *Trends Cell Biol.* 13:289–294.

Blasco, M.A., H.W. Lee, M.P. Hande, E. Samper, P.M. Lansdorp, R.A. DePinho, C.W. Greider. 1997. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell*. 91:25–34.

Braun, K.M., C. Niemann, U.B. Jensen, J.P. Sundberg, V. Silva-Vargas, and F.M. Watt. 2003. Manipulation of stem cell proliferation and lineage commitment: visualisation of label-retaining cells in wholemounts of mouse epidermis. *Development*. 130:5241–5255.

Canela, A., E. Vera, P. Klatt, and M.A. Blasco. 2007. High-throughput telomere length quantification by FISH and its application to human population studies. *Proc. Natl. Acad. Sci. USA*. 104:5300–5305.

Cawthon, R.M., K.R. Smith, E. O'Brien, A. Sivatchenko, and R.A. Kerber. 2003. Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet*. 361:393–395.

Chan, S.W., and E.H. Blackburn. 2002. New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin. *Oncogene*. 21:553–563.

Collins, K., and J.R. Mitchell. 2002. Telomerase in the human organism. *Oncogene*. 21:564–579.

Cotsarelis, G., T.T. Sun, and R.M. Lavker. 1990. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell*. 61:1329–1337.

de Lange, T. 2005. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev.* 19:2100–2110.

Epel, E.S., E.H. Blackburn, J. Lin, F.S. Dhabhar, N.E. Adler, J.D. Morrow, and R.M. Cawthorn. 2004. Accelerated telomere shortening in response to life stress. *Proc. Natl. Acad. Sci. USA*. 101:17312–17315.

Ferron, S., H. Mira, S. Franco, M. Cano-Jaimez, E. Bellmunt, C. Ramirez, I. Farinas, and M.A. Blasco. 2004. Telomere shortening and chromosomal instability abrogates proliferation of adult but not embryonic neural stem cells. *Development*. 131:4059–4070.

Flores, I., M.L. Cayuela, and M.A. Blasco. 2005. Effects of telomerase and telomere length on epidermal stem cell behavior. *Science*. 309:1253–1256.

Flores, I., R. Benetti, and M.A. Blasco. 2006. Telomerase regulation and stem cell behaviour. *Curr. Opin. Cell Biol.* 18:254–260.

Fuchs, E., T. Tumbar, and G. Guasch. 2004. Socializing with the neighbours: stem cells and their niche. *Cell*. 116:769–778.

García-Cao, I., M. García-Cao, A. Tomás-Loba, J. Martín-Caballero, J.M. Flores, P. Klatt, M.A. Blasco, and M. Serrano. 2006. Increased p53 activity does not accelerate telomere-driven aging. *EMBO Rep.* 7:546–552.

Gonzalez-Suarez, E., E. Samper, J.M. Flores, and M.A. Blasco. 2000. Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis. *Nat. Genet.* 26:114–117.

Greenberg, R.A., L. Chin, A. Femino, K.H. Lee, G.J. Gottlieb, R.H. Singer, C.W. Greider, and R.A. DePinho. 1999. Short dysfunctional telomeres impair tumorigenesis in the INK4a(delta2/3) cancer-prone mouse. *Cell*. 97:515–525.

Harley, C.B., A.B. Futcher, and C.W. Greider. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature*. 345:458–460.

Harrington, L. 2004. Does the reservoir for self-renewal stem from the ends? *Oncogene*. 23:7283–7289.

Hemann, M.T., M.A. Strong, L.Y. Hao, and C.W. Greider. 2001. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell*. 107:67–77.

Herrera, E., E. Samper, J. Martin-Caballero, J.M. Flores, H.W. Lee, and M.A. Blasco. 1999. Disease states associated to telomerase deficiency appear earlier in mice with short telomeres. *EMBO J.* 18:2950–2960.

Lee, H.-W., M.A. Blasco, G.J. Gottlieb, C.W. Greider, and R.A. DePinho. 1998. Essential role of mouse telomerase in highly proliferative organs. *Nature*. 392:569–574.

Leri, A., S. Franco, A. Zacheo, L. Barlucchi, S. Chimenti, F. Limana, B. Nadal-Ginard, J. Kajstura, P. Anversa, and M.A. Blasco. 2003. Ablation of telomerase and telomere loss leads to cardiac dilatation and heart failure associated with p53 upregulation. *EMBO J.* 22:131–139.

Marrone, A., D. Stevens, T. Vulliamy, I. Dokal, and P.J. Mason. 2004. Heterozygous telomerase RNA mutations found in dyskeratosis congenita and aplastic anemia reduce telomerase activity via haploinsufficiency. *Blood*. 104:3936–3942.

Mason, P.J., D.B. Wilson, and M. Bessler. 2005. Dyskeratosis congenita—a disease of dysfunctional telomere maintenance. *Curr. Mol. Med.* 5:159–170.

Mitchell, J.R., E. Wood, and K. Collins. 1999. A telomerase component is defective in the human disease dyskeratosis congenita. *Nature*. 402:551–555.

Moore, K.A., and I.R. Lemischka. 2006. Stem cells and their niches. *Science*. 311:1880–1885.

Morris, R.J., Y. Liu, L. Marles, Z. Yang, C. Trempus, S. Li, J.S. Lin, J.A. Sawicki, and G. Cotsarelis. 2004. Capturing and profiling adult hair follicle stem cells. *Nat. Biotechnol.* 22:411–417.

Muñoz, P., R. Blanco, J.M. Flores, and M.A. Blasco. 2005. XPF nuclease-dependent telomere loss and increased DNA damage in mice overexpressing TRF2 result in premature aging and cancer. *Nat. Genet.* 37:1063–1071.

Oshima, H., A. Rochat, C. Kedzia, K. Kobayashi, and Y. Barrandon. 2001. Morphogenesis and renewal of hair follicles from adult multipotent item cells. *Cell*. 104:233–245.

Rudolph, K.L., S. Chang, H.-W. Lee, M.A. Blasco, G. Gottlieb, C.W. Greider, and R.A. DePinho. 1999. Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell*. 96:701–712.

Samani, N.J., R. Boultby, R. Butler, J.R. Thompson, and A.H. Goodall. 2001. Telomere shortening in atherosclerosis. *Lancet*. 358:472–473.

Samper, E., F.A. Goytisolo, P. Slijepcevic, P.P. van Buul, and M.A. Blasco. 2000. Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and the G-strand overhang. *EMBO Rep.* 1:244–252.

Samper, E., J.M. Flores, and M.A. Blasco. 2001. Restoration of telomerase activity rescues chromosomal instability and premature aging in Terc−/− mice with short telomeres. *EMBO Rep.* 2:800–807.

Samper, E., P. Fernandez, R. Equia, L. Martin-Rivera, A. Bernad, M.A. Blasco, and M. Aracil. 2002. Long-term repopulating ability of telomerase-deficient murine hematopoietic stem cells. *Blood*. 99:2767–2775.

Sarin, K.Y., P. Cheung, D. Gilison, E. Lee, R.I. Tennen, E. Wang, M.K. Artandi, A.E. Oro, and S.E. Artandi. 2005. Conditional telomerase induction causes proliferation of hair follicle stem cells. *Nature*. 436:1048–1052.

Teixeira, M.T., M. Arneric, P. Sperisen, and J. Lingner. 2004. Telomere length homeostasis is achieved via a switch between telomerase-extendible and -nonextendible states. *Cell*. 117:323–335.

Tsakiri, K.D., J.T. Cronkhite, P.J. Kuan, C. Xing, G. Raghu, J.C. Weissler, R.L. Rosenblatt, J.W. Shay, and C.K. Garcia. 2007. Adult-onset pulmonary fibrosis caused by mutations in telomerase. *Proc. Natl. Acad. Sci. USA*. 104:7552–7557.

Tumbar, T., G. Guasch, V. Greco, C. Blanpain, W.E. Lowry, M. Rendl, and E. Fuchs. 2004. Defining the epithelial stem cell niche in skin. *Science*. 303:359–363.

Valdes, A.M., T. Andrew, J.P. Gardner, M. Kimura, E. Oelsner, L.F. Cherkas, A. Aviv, and T.D. Spector. 2005. Obesity, cigarette smoking, and telomere length in women. *Lancet*. 366:662–664.

Vulliamy, T., A. Marrone, F. Goldman, A. Dearlove, M. Bessler, P.J. Mason, and I. Dokal. 2001. The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. *Nature*. 413:432–435.

Vulliamy, T., A. Marrone, R. Szydlo, A. Walne, P.J. Mason, and I. Dokal. 2004. Disease anticipation is associated with progressive telomere shortening in families with dyskeratosis congenita due to mutations in TERC. *Nat. Genet.* 36:447–449.

Wiemann, S.U., A. Satyanarayana, M. Tsahurida, H.L. Tillman, L. Zender, J. Klempnauer, P. Flemming, S. Franco, M.A. Blasco, M.P. Manns, K.L. Rudolph. 2002. Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis. *FASEB J.* 16:935–942.

Wilson, C., G. Cotsarelis, Z.G. Wei, E. Fryer, J. Margolis-Fryer, M. Ostead, R. Tokarek, T.T. Sun, and R.M. Lavker. 1994. Cells within the bulge region of mouse hair follicle transiently proliferate during early anagen: heterogeneity and functional differences of various hair cycles. *Differentiation*. 55:127–136.

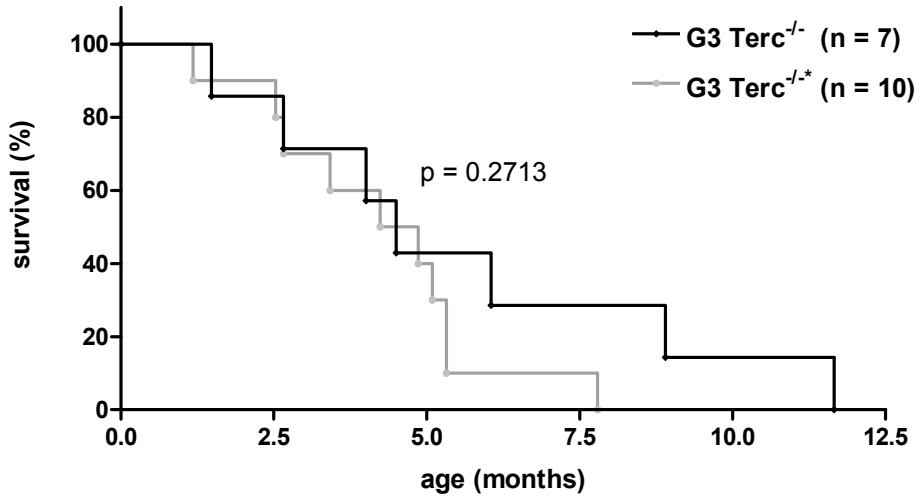
Yamaguchi, H., R.T. Calado, H. Ly, S. Kaijigaya, G.M. Baerlocher, S.J. Chanock, P.M. Lansdorp, and N.S. Young. 2005. Mutations in TERT, the gene for telomerase reverse transcriptase, in aplastic anemia. *N. Engl. J. Med.* 352:1413–1424.

Zijlmans, J.M., U.M. Martens, S.S. Poon, A.K. Raap, H.J. Tanke, R.K. Ward, and P.M. Lansdorp. 1997. Telomeres in the mouse have large inter-chromosomal variations in the number of T2AG3 repeats. *Proc. Natl. Acad. Sci. USA*. 94:7423–7428.

9.4. Annex

Comparation of the survival of late generation telomerase-deficient mice (G3 Terc$^{-/-}$) to late generation telomerase-deficient mice (G3 Terc$^{-/-*}$) generated from the crosses of heterozygous mice with late generation telomerase-deficient mice (G2 Terc$^{-/-}$).

We crossed late generation telomerase-deficient mice with short telomeres with heterozygous mice with a normal telomere length resulting in late generation telomerase-deficient offspring (G3 Terc$^{-/-*}$) that has one set of chromosomes with normal telomeres whereas the other set remained with critically short telomeres and their littermates (G3 Terc$^{+/-*}$), reconstituted with one allele of the terc gene, in which all chromosomes have detectable telomeres [65]. Now the question arises if, containing one set of chromosomes with telomeres of a normal length, like it is the case for the G3 Terc$^{-/-*}$ mice, would prolong the short life span of these mice comparing them to "normal" G3 Terc$^{-/-}$ mice. Our results indicate that there is no significant difference in the survival of these two genotypes (Fig. 1) ruling out the possibility of an advantage in survival after re-introduction of a set of chromosomes with a normal telomere length from the heterozygous parent.



Annex figure 1: No significant difference in the survival indicated by the Kaplan-Meyer survival curve of mice of the indicated genotype. *(n)* Number of mice of each genotype. Statistical comparison between genotypes using the log rank test is shown.

10. SECOND PROJECT

10.1. INTRODUCTION: Deficient mismatch repair improves organismal fitness and survival of mice with dysfunctional telomeres

Mismatch repair (MMR) is a DNA repair system that, in addition to the removal of errors associated with DNA replication, has a role in meiotic and mitotic recombination. MMR functions to inhibit recombination between non-identical sequences. MMR also has additional functions in the signaling of DNA-damage, in the generation of antibody diversity through class-switch recombination and somatic hypermutation, and a, less well-defined, function in triplet-repeat expansion [157]. The known function of MMR proteins in repressing recombination between nonhomologous sequences suggested that, in the absence of MMR, telomere elongation is more likely to proceed by a recombination-based alternative mechanism (ALT).

Rizki and Lundblad (2001) compared the proliferative potential of budding yeast lacking active telomerase to yeast with defects in the mismatch-repair machinery, and to yeast with defects in both systems. They showed that doubly-deficient yeast cells possessed an enhanced survival when compared to yeast lacking telomerase alone. The explanation for this phenomenon was that homologous recombination between non-identical sequences was no longer inhibited and alternative telomere lengthening could take place [13]. Bechter et al. (2004) investigated a human cancer cell line with a MMR defect. In a similar finding, inhibition of telomerase in these cells resulted in telomerase-independent, ALT-like telomere elongation [14]. Additionally, several papers have reported a role for MMR proteins in the signaling of DNA damage [16, 17, 175]. MMR proteins were shown to be involved in the p53 pathway and the ATR/ATM-mediated DNA damage signaling pathway, which was activated in response to increased DNA replication errors due to MMR deficiency [16, 17, 175, 178].

We generated mice doubly deficient for telomerase and the MMR gene *pms2* to examine their combined effects on cancer and aging.

Personal contribution:
I performed all experiments, except for the western blot analysis of the DNA damage pathway in mouse embryonic fibroblasts, which was performed by Purification Munoz. Juana Flores analyzed the pathologies of the mice.

10.2. INTRODUCCIÓN: Una reparación deficiente de los errores de apareamiento mejora el estado de forma del organismo y la supervivencia de ratones con telómeros no funcionales

La reparación de apareamientos erróneos (MMR) es un sistema de reparación del ADN que, además de remover los errores asociados a la replicación del ADN, juega un rol en la recombinación, tanto mitótica como meiótica; MMR inhibe la recombinación entre secuencias no idénticas. MMR tiene también funciones adicionales en la señalización del daño al ADN, en la generación de la diversidad de anticuerpos a través de la recombinación de cambio de clase e hipermutación somática y, finalmente, un rol algo menos definido en la expansión de repeticiones de tripletes [157]. La función conocida de las proteínas del MMR en la represión de la recombinación entre secuencias sin homología ha sugerido que, en ausencia de MMR, es más probable que la elongación telomérica ocurra mediante un mecanismo alternativo basado en la recombinación (ALT).

Rizki y Lundblad (2001) compararon el potencial proliferativo de levaduras gemantes, carentes de actividad de telomerasa, con levaduras con defectos en la maquinaria de reparación de apareamientos erróneos, y con levaduras con defectos en los dos sistemas. Ellos demostraron que las levaduras doblemente deficientes poseían una sobrevida aumentada al ser comparadas con las que sólo eran deficientes en telomerasa. La explicación para esto fue que la recombinación homóloga entre secuencias no idénticas ya no era inhibida, y así podía tener lugar un alargamiento alternativo de los telómeros [13]. Bechter *et al*. (2004) investigaron una línea de células cancerosas humanas con un defecto en el MMR. En un descubrimiento similar, la inhibición de la telomerasa en estas células resultó en una elongación de los telómeros independiente de telomerasa, es decir, tipo ALT [14]. Además, varios artículos han reportado un rol de las proteínas del MMR en la señalización del daño al ADN [16, 17, 175]. Se ha demostrado también que las proteínas del MMR están involucradas en la vía de p53 y la vía de señalización del daño al ADN mediada por ATR/ATM, la cual es activada en respuesta a un aumento en los errores de replicación del ADN debido a la deficiencia en el MMR [16, 17, 175, 178].

Nosotros hemos generado ratones doblemente deficientes en telomerasa y el gen *pms2* del sistema de MMR para examinar sus efectos combinados sobre el cáncer y el envejecimiento.

Contribución personal:
Todos los experimentos han sido realizados por mí, excepto el análisis mediante *western blot* de la vía de señalización del daño al ADN en fibroblastos embriónicos de ratón, los cuales fueron realizados por Purificación Muñoz. Juana Flores analizó las patologías de los ratones.

10.3. PUBLICATION

Siegl-Cachedenier I, Muñoz P, Flores JM, Klatt P, Blasco MA.

Deficient mismatch repair improves organismal fitness and survival of mice with dysfunctional telomeres.

Genes Dev. 2007 Sep 1;21(17):2234-47.

Deficient mismatch repair improves organismal fitness and survival of mice with dysfunctional telomeres

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Mismatch repair (MMR) has important roles in meiotic and mitotic recombination, DNA damage signaling, and various aspects of DNA metabolism including class-switch recombination, somatic hypermutation, and triplet-repeat expansion. Defects in MMR are responsible for human cancers characterized by microsatellite instability. Intriguingly, MMR deficiency has been shown to rescue survival and proliferation of telomerase-deficient yeast strains. A putative role for MMR at mammalian telomeres that could have an impact on cancer and aging is, however, unknown. Here, we studied the role of MMR in response to dysfunctional telomeres by generating mice doubly deficient for telomerase and the PMS2 MMR gene (*Terc*$^{-/-}$*/PMS2*$^{-/-}$ mice). PMS2 deficiency prolonged the mean lifespan and median survival of telomerase-deficient mice concomitant with rescue of degenerative pathologies. This rescue of survival was independent of changes in telomere length, in sister telomere recombination, and in microsatellite instability. Importantly, PMS2 deficiency rescued cell proliferation defects but not apoptotic defects in vivo, concomitant with a decreased p21 induction in response to short telomeres. The proliferative advantage conferred to telomerase-deficient cells by the ablation of PMS2 did not produce increased tumors. Indeed, *Terc*$^{-/-}$*/PMS2*$^{-/-}$ mice showed reduced tumors compared with *PMS2*$^{-/-}$ mice, in agreement with a tumor suppressor role for short telomeres in the context of MMR deficiencies. These results highlight an unprecedented role for MMR in mediating the cellular response to dysfunctional telomeres in vivo by attenuating p21 induction.

[*Keywords*: Telomeres; MMR; PMS2; telomerase knockout mice; aging; cancer]

Supplemental material is available at http://www.genesdev.org.

Received February 22, 2007; revised version accepted July 11, 2007.

Mismatch repair (MMR) is a multifaceted DNA repair system with a crucial role in removing errors associated with DNA replication (for review, see Jiricny 2006). In addition, MMR inhibits recombination between nonidentical sequences and influences a number of other processes associated with DNA metabolism, including DNA-damage signaling, class-switch recombination, somatic hypermutation, and triplet-repeat expansion (for review, see Jiricny 2006). Loss of MMR results in the so-called "mutator phenotype," which can lead to increased susceptibility to cancer (Fishel et al. 1993; Leach et al. 1993; Bronner et al. 1994; Nicolaides et al. 1994; Papadopoulos et al. 1994; Shibata et al. 1994). Indeed, a number of human tumors including cancers of the colon and the endometrium show a phenotype known as "microsatellite instability," which is associated with defects in the MMR pathway (for review, see Modrich 1994). The mammalian MMR machinery is comprised of six homologs of the *Escherichia coli* MutS family of genes (MSH genes: MSH1 to MSH6) and four homologs of the MutL family of genes (MLH genes: MLH1, MLH3, PMS1, and PMS2). Mutations in both families of genes have been associated with human hereditary nonpolyposis colon cancer (Fishel et al. 1993; Leach et al. 1993; Bronner et al. 1994; Nicolaides et al. 1994; Papadopoulos et al. 1994). Mice deficient for different MMR genes show increased spontaneous mutation as well as an increased susceptibility to develop cancer, with MSH2 knockout mice showing the most severe phenotype (Baker et al. 1995; de Wind et al. 1995; Prolla et al. 1998). A role for MMR proteins in DNA damage signaling, which in turn could affect tumorigenesis, has also been proposed (Duckett et al. 1999; Peters et al. 2003; Luo et al. 2004). In particular, MMR proteins have been directly involved in signaling DNA damage through the p53 pathway (Duckett et al. 1999; Peters et al. 2003; Luo et al. 2004); in turn, DNA

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Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.430107.

replication errors produced by MMR deficiency have been proposed to result in ATR/ATM-mediated DNA damage signaling (Brown et al. 2003).

Telomeres are protective structures at the ends of chromosomes that consist of tandem TTAGGG repeats and associated proteins (Chan and Blackburn 2002; de Lange 2005). Critical telomere shortening and loss of function of telomere-binding proteins results in loss of telomere protection, end-to-end chromosome fusions, and cell cycle arrest or apoptosis (van Steensel et al. 1998; Goytisolo and Blasco 2002; de Lange 2005). Telomere shortening is envisioned as a potent tumor suppressor mechanism (González-Suárez et al. 2000; Blasco and Hahn 2003; Blasco 2005). Telomerase activity, in turn, is able to elongate telomeres in those cells where it is highly expressed, such as in the vast majority of human cancers, and it is associated with cell immortalization (Shay and Wright 2006). Mice that lack telomerase activity show premature loss of organismal viability when their telomeres become critically short and are resistant to cancer (Lee et al. 1998; Greenberg et al. 1999; Herrera et al. 1999; González-Suárez et al. 2000), with the only exceptions being p53-deficient and TRF2-overexpressing genetic backgrounds (Chin et al. 1999; Artandi et al. 2000; Blanco et al. 2007).

Intriguingly, previous work in budding yeast indicated that MMR abrogation rescued survival of telomerase-deficient strains (Rizki and Lundblad 2001). This rescue was attributed to the known role of MMR in repressing recombination between nonhomologous sequences, which in turn could favor survival in the absence of telomerase by sustaining recombination-based alternative elongation of telomeres, or ALT mechanisms. A putative role for MMR in mammalian telomere biology is unknown, however, except for the description of increased telomeric recombination in MMR-deficient human colon cancer cells (Bechter et al. 2004).

Here, we set out to address whether MMR-deficiency had any impact on survival and life span of telomerase-deficient mice by generating mice doubly deficient for telomerase and the MMR gene PMS2 (*Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice). PMS2-deficient mice have been previously shown to have a decreased MMR efficiency, increased microsatellite instability, as well as an increased susceptibility to develop sarcomas and lymphomas (Baker et al. 1995; Prolla et al. 1998). In addition, PMS2-deficient males are infertile due to abnormal chromosome synapsis in meiosis (Baker et al. 1995). Interestingly, PMS2 deficiency rescued median survival and lifespan of telomerase-deficient mice. This rescue in survival was concomitant with a decreased incidence of degenerative pathologies and, at the cellular level, with an increased proliferative potential. Interestingly, these effects were independent of telomere length and of the presence of severe telomere dysfunction as indicated by similar frequencies of end-to-end fusions, critically short telomeres, and γH2AX-positive cells. Similarly, the rescue of phenotypes associated with dysfunctional telomeres was not associated with significant differences in the frequency of homologous recombination events between sister telomeres (telomere sister chromatid exchanges, T-SCE), suggesting that PMS2 deficiency is not rescuing survival by favoring the activation of mammalian ALT pathways (Dunham et al. 2000; Muntoni and Reddel 2005). Similarly, we did not find significant differences in microsatellite instability between *PMS2*$^{-/-}$ and *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice, which could explain the rescue in survival. Importantly, we found an attenuated p21 induction both in vivo and in vitro in mice and cells doubly deficient for telomerase and PMS2 compared with the single telomerase-deficient controls, which was concomitant with increased proliferation rates in vivo as well as in vitro. This decreased p21 response was paralleled by decreased p53 levels. All together, these results suggest that MMR deficiency rescues organismal survival and proliferation in telomerase-deficient mice by attenuating the anti-proliferative response associated with short telomeres.

Results

PMS2 deficiency rescues median survival and mean lifespan of mice with dysfunctional telomeres

To study the impact of PMS2 deficiency in *Terc*$^{-/-}$ mouse survival, we generated increasing generations of double mutant *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice as well as of the single *Terc*$^{-/-}$ controls (see Materials and Methods). In a C57BL6 genetic background, *Terc*$^{-/-}$ mice can be maintained for up to three generations (G1 to G3). First, we confirmed that PMS2-deficient mice have a decreased median survival compared with wild-type mice (Baker et al. 1995; $P < 0.001$; Fig. 1), and that increasing generations of telomerase-deficient mice show progressively decreased median survival compared with wild-type controls (Fig. 1; $P < 0.005$ for all comparisons between wild-type and G1–G3 *Terc*$^{-/-}$ mice; see "statistics" in Supplemental Material for comparisons between mouse generations; Herrera et al. 1999). PMS2 abrogation extended median survival of telomerase-deficient mice in G1 and G2 mouse generations, and this difference reached statistical significance when comparing G2 *Terc*$^{-/-}$/*PMS2*$^{-/-}$ with single G2 *Terc*$^{-/-}$ mice ($P < 0.001$; Fig. 1). In addition, PMS2 deficiency reproducibly increased both median survival as well as mean lifespan of increasing generations of telomerase-deficient mice, and these differences reached statistical significance when comparing G2 *Terc*$^{-/-}$/*PMS2*$^{-/-}$ with single G2 *Terc*$^{-/-}$ mice ($P < 0.001$; Supplemental Fig. 1A,B). The fact that the G3 *Terc*$^{-/-}$/*PMS2*$^{-/-}$ to G3 *Terc*$^{-/-}$ comparisons did not reach statistical significance is probably due to the small numbers of mice obtained for this generation due to severe infertility of G2 *Terc*$^{-/-}$ mice (Herrera et al. 1999). Significantly, G1 and G2 *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice showed increased median survival and mean lifespan compared with single *PMS2*$^{-/-}$ mice ($P < 0.05$ in both cases; Supplemental Fig. 1A,B), also suggesting that telomerase deficiency partially ameliorates PMS2-associated loss of organismal survival (see later). All together, these results suggest that PMS2 has a role in the organismal response to critically short and dysfunctional telomeres associ-

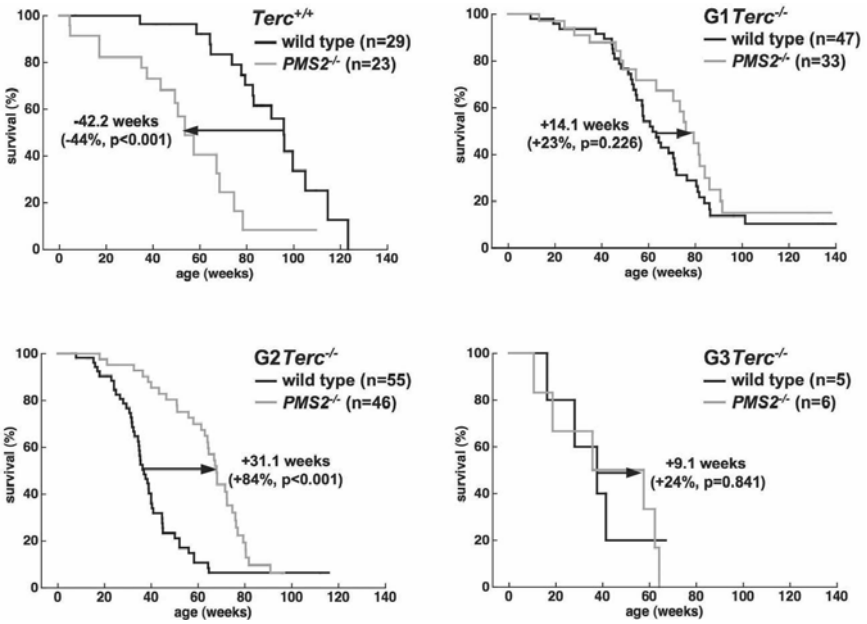

Figure 1. PMS2 deficiency rescues organismal survival of mice with dysfunctional telomeres. Kaplan–Meyer survival curve of mice of the indicated genotype. (*n*) Number of mice of each genotype. Statistical comparisons between genotypes using the log rank test are also shown. For statistical comparisons between mouse generations, see "statistics" in the Supplemental Material.

ated with telomerase deficiency. Of note, this is the second genetic alteration shown to rescue life span and survival of telomerase-deficient mice after a recent publication describing that p21 abrogation has a similar effect (Choudhury et al. 2007).

PMS2 deficiency rescues degenerative pathologies in mice with dysfunctional telomeres without accelerating carcinogenesis

Next, we performed a full histopathological analysis to determine the cause of death of the different mouse cohorts. As previously described, single PMS2-deficient mice showed an increased cancer susceptibility, with a high incidence of lymphomas (55.6%), followed by histiocytic sarcomas (22%; liver, lung, kidney) and colon carcinomas (11.1%) (Fig. 2A; Baker et al. 1995). Similarly, PMS2 abrogation also increased the incidence of these tumors in the context of *Terc*-deficient mice ($P < 0.001$ for all comparisons except G3; Fig. 2A). However, increasing generations of $Terc^{-/-}/PMS2^{-/-}$ mice showed a progressively reduced incidence of tumors compared with the single $PMS2^{-/-}$ mice. In particular, lymphomas were reduced from 55% in the single $PMS2^{-/-}$ mice to only 25% in the G3 $Terc^{-/-}/PMS2^{-/-}$ (Fig. 2A). Similarly, histiocytic sarcomas and carcinomas were also decreased with increasing $Terc^{-/-}/PMS2^{-/-}$ mouse generations compared with the $PMS2^{-/-}$ controls (Fig. 2A). These differences were statistically significant when comparing all malignancies in G2–G3 $Terc^{-/-}/PMS2^{-/-}$ mice with the single $PMS2^{-/-}$ controls ($P < 0.05$ for all comparisons; see "statistics" in Supplemental Material). Single *Terc*-deficient mice also showed decreased tumors with increasing mouse generations (Fig. 2A; $P = 0.028$ when comparing wild-type and G2 $Terc^{-/-}$ mice; see "statistics" in Supplemental Material). These results are in agreement with short telomeres acting as potent tumor suppressors in the context of telomerase deficiency (González-Suárez et al. 2000; Blasco and Hahn 2003). Finally, these results indicate that telomerase deficiency and short telomeres suppress PMS2-induced tumorigenesis, which in turn could contribute to the increased lifespan of G1 and G2 $Terc^{-/-}/PMS2^{-/-}$ mice compared with single *PMS2*-mutant mice, as well as suggest that telomerase inhibitors would be effective in ceasing the growth of *PMS2*-mutant tumors.

In agreement with previous reports, single *Terc*-deficient mice showed an increased incidence of various degenerative pathologies in the different mouse generations that was not observed in $PMS2^{-/-}$ mice, intestinal atrophies being the most abundant lesions (Supplemental Fig. 2; Lee et al. 1998; Herrera et al. 1999). In particular, the incidence of severe intestinal lesions (see Materials and Methods for detailed description of these lesions) was increased from 30.8% in G1 $Terc^{-/-}$ to 75% in G3 $Terc^{-/-}$ mice (Fig. 2B; $P < 0.001$ when comparing wild-type mice with each $Terc^{-/-}$ generation; see "statistics" in Supplemental Material). Remarkably, PMS2 deficiency rescued degenerative pathologies in every $Terc^{-/-}/PMS2^{-/-}$ mouse generation compared with the single $Terc^{-/-}$ mice ($P < 0.05$ for all comparisons; Fig. 2B). These results suggest that PMS2 abrogation ameliorates degenerative pathologies associated with short telomeres, in agreement with the fact that PMS2 deficiency rescues both median life span and survival of telomerase-deficient mice (Fig. 1).

PMS2 deficiency rescues proliferation defects but not apoptosis in telomerase-deficient mice with short telomeres

Decreased survival of late-generation Terc-deficient mice has been previously shown to be the consequence

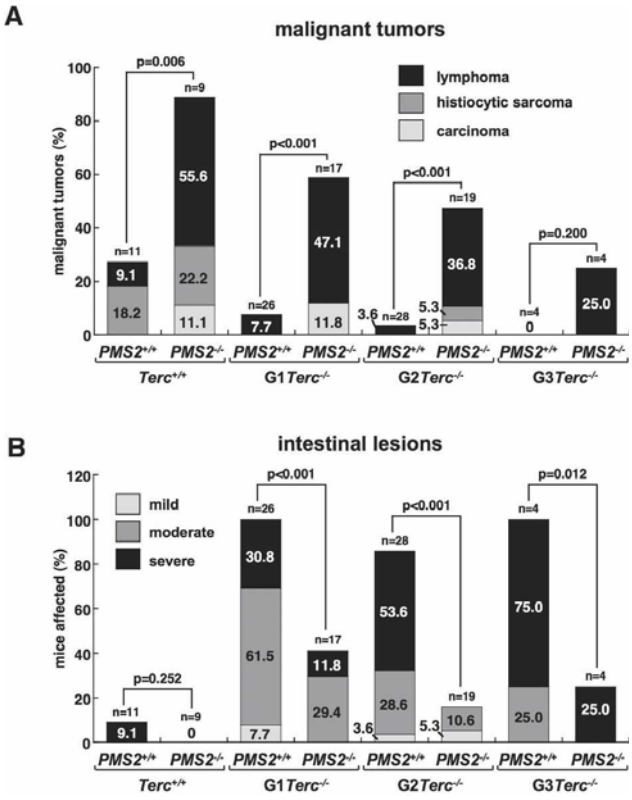

Figure 2. Rescue of malignant tumors and degenerative pathologies in *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice. (*A*) Percentage of mice of the indicated genotype showing malignant tumors (lymphoma, histiocytic sarcoma, carcinoma) at the time of death. Statistical comparisons using the χ^2 test are also shown. For statistical comparisons between mouse generations, see "statistics" in the Supplemental Material. (*B*) Percentage of mice of the indicated genotype showing mild, moderate, and severe intestinal atrophy at the time of death (see Materials and Methods for description of the lesions). Statistical comparisons using the χ^2 test are also shown. For statistical comparisons between mouse generations, see "statistics" in the Supplemental Material.

of severe proliferative defects and increased apoptosis in tissues characterized by high regeneration rates, such as the gastrointestinal (GI) tract (Lee et al. 1998; Herrera et al. 1999). Indeed, according to histopathological analysis of *Terc*-deficient moribund mice (Supplemental Fig. 2), the GI tract was the most frequently affected organ. For this reason we focused our analysis at the cellular level on the intestine. First, we confirmed that G1 to G3 *Terc*-deficient mice showed increased apoptosis compared with wild-type controls in the GI tract (Fig. 3A,B; $P < 0.005$ for all comparisons between generations in "statistics" in Supplemental Material). This increased apoptosis, however, was not significantly rescued by PMS2 deficiency with increasing generations of *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice compared with the single *Terc*$^{-/-}$ controls (Fig. 3A,B), indicating that PMS2 abrogation does not rescue degenerative pathologies and mouse survival by decreasing apoptotic rates in *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice. Indeed, apoptosis was significantly increased in late generation G3 *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice compared with the single G3 *Terc*$^{-/-}$ controls ($P = 0.04$; Fig. 3B). In marked contrast, we observed a significant rescue of proliferative defects in the GI tract of different generation *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice compared with the single Terc-deficient controls ($P \leq 0.01$ for all comparisons; Fig. 3A,B), suggesting that PMS2 deficiency specifically rescues proliferation defects but not apoptosis associated with dysfunctional telomeres. This rescue of proliferation defects may explain the rescue of degenerative pathologies and survival in *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice. This is analogous to what has been recently described for *Terc*$^{-/-}$/*p21*$^{-/-}$ mice, in which p21 deficiency rescued survival and proliferative defects but not apoptotic defects associated with ablation of telomerase (Choudhury et al. 2007). Finally, several mechanisms can be envisioned by which PMS2 deficiency may be rescuing proliferative defects and organismal survival associated with dysfunctional telomeres. On one hand, PMS2 deficiency may be rescuing telomere length or telomere capping defects associated with *Terc* deficiency. In this regard, PMS2 abrogation may be favoring telomere recombination events and therefore activation of ALT pathways. This would be in agreement with its known role in inhibiting mitotic recombination (Modrich 1994; Jiricny 2006), similarly to that previously proposed for simultaneous telomerase and MMR deficiency in yeast (Rizki and Lundblad 2001). Alternatively, PMS2 may have a prominent role in the cellular response to short dysfunctional telomeres, resulting in a rescue of proliferative defects, similarly to that recently described for p21 deficiency (Choudhury et al. 2007).

PMS2 deficiency promotes cellular immortalization independently of telomere length

To address whether the improved survival of *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice compared with single *Terc*$^{-/-}$ mice was associated with changes in telomere length caused by *PMS2* deficiency, we first measured telomere length using quantitative fluorescence in situ hybridization, Q-FISH, on metaphase spreads from primary (passage 2) littermate mouse embryonic fibroblasts (MEF) of the different genotypes performed in parallel (see Materials and Methods; numbers in Fig. 4A refer to individual littermate MEF). We found a similar rate of telomere loss with increasing generations (G1 to G3) of single *Terc*$^{-/-}$ or double *Terc*$^{-/-}$/*PMS2*$^{-/-}$ MEF ($P < 0.05$ for all comparisons between G1–G3 *Terc*$^{-/-}$ or double *Terc*$^{-/-}$/*PMS2*$^{-/-}$ littermate mice and the corresponding Terc$^{+/+}$ controls; Fig. 4A), indicating that PMS2 deficiency does not impact telomere length regulation. Furthermore, in agreement with telomere shortening along increasing generations of telomerase deficiency, we detected increased frequencies of very short telomeres (<5 kb) in single G1–G3 *Terc*$^{-/-}$ MEF ($P < 0.001$ for all comparisons between generations; Supplemental Fig. 3), which were further increased in the corresponding double mutant *Terc*$^{-/-}$/*PMS2*$^{-/-}$ MEF ($P < 0.001$ for comparisons between G1 and G2 *Terc*$^{-/-}$ MEF with the corresponding double *Terc*$^{-/-}$/*PMS2*$^{-/-}$ MEF; Supplemental Fig. 3), again ruling

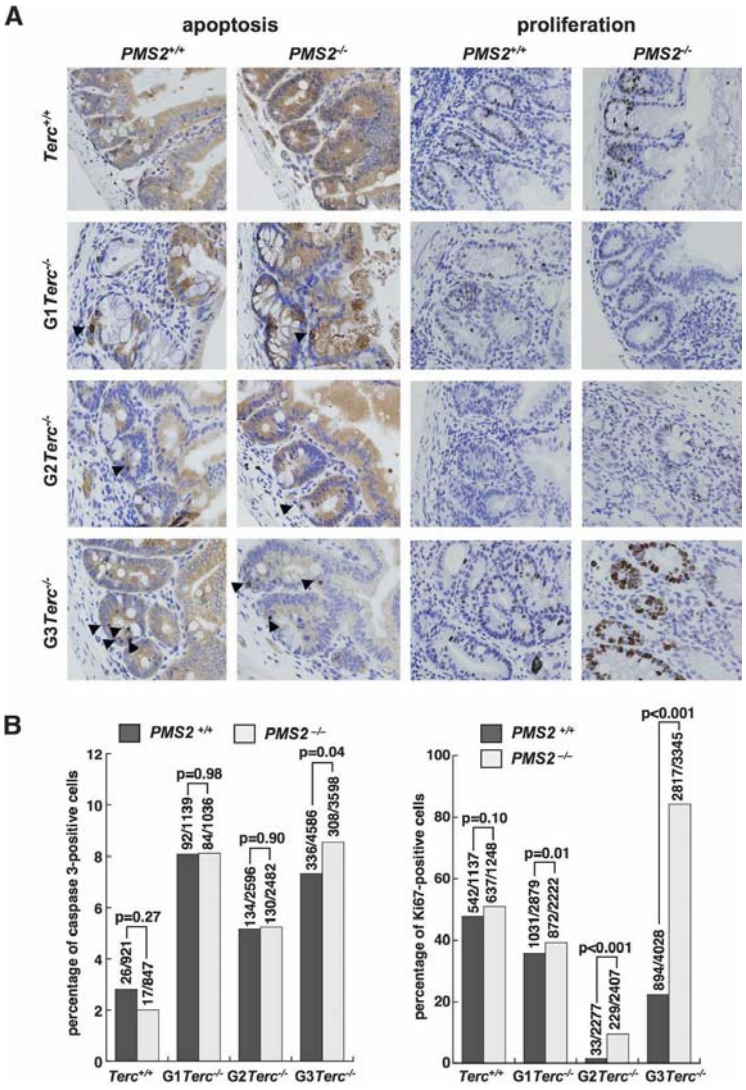

Figure 3. Rescue of proliferation defects but not apoptosis in $Terc^{-/-}/PMS2^{-/-}$ mice. (*A*) Representative examples of sections of the small intestine from mice of the indicated genotypes stained for caspase 3 as a marker for apoptosis (*left*) and Ki67 as marker for proliferating cells (*right*). Note similar numbers of caspase 3-positive cells in single G3 $Terc^{-/-}$ and double mutant G3$Terc^{-/-}/PMS2^{-/-}$ mice. (Arrows) Caspase 3-positive cells. Note the increase of Ki67-positive cells (brown staining) in double mutant G3 $Terc^{-/-}/PMS2^{-/-}$ mice compared with the single G3 $Terc^{-/-}$ controls. (*B*) Quantification of percentage of cells showing caspase 3- and Ki67-positive staining at the GI tract in mice of the indicated genotypes. The total number of cells scored for the analysis is also indicated on *top* of each bar. Statistical comparisons using the χ^2 test are also shown.

out that the increased survival of $Terc^{-/-}/PMS2^{-/-}$ mice is due to a rescue of critically short telomeres in the absence of PMS2. The fact that the frequency of very short telomeres (<5 kb) was increased in double mutant $Terc^{-/-}/PMS2^{-/-}$ MEF compared with the single $Terc^{-/-}$ MEF may be the consequence of increased proliferation rates in the absence of PMS2 (see Fig. 3A,B), which in turn may result in increased telomere erosion. Finally, these telomere length results obtained with littermate MEF were confirmed by increasing the number of MEF per genotype (Supplemental Fig. 4). Furthermore, they were also confirmed by using Southern-based "terminal restriction fragment" (TRF) analysis (Supplemental Fig. 5; Blasco et al. 1997).

These results with cultured MEF were also recapitulated when measuring telomere length using Q-FISH directly on small intestine sections (see Materials and Methods; González-Suárez et al. 2000; Muñoz et al. 2005). Again, we found a similar rate of telomere loss with increasing generations (G1 to G2) of single $Terc^{-/-}$ or double $Terc^{-/-}/PMS2^{-/-}$ MEF ($P < 0.0001$ for all comparisons between G1–G2 $Terc^{-/-}$ or double $Terc^{-/-}/PMS2^{-/-}$ mice and the corresponding $Terc^{+/+}$ controls; Fig. 4B for quantification and Fig. 4C for representative images), indicating that PMS2 deficiency does not rescue telomere length in the in vivo setting of the $Terc^{-/-}$ small intestine.

We next studied whether *PMS2* deficiency could rescue in vitro proliferative defects in late-generation $Terc^{-/-}$ MEF. In particular, we have previously shown that late-generation primary (passage 2) $Terc^{-/-}$ MEF showed decreased immortalization ability in culture compared with wild-type controls (Espejel and Blasco 2002). To this end, we studied the spontaneous immortalization ability of increasing generation (G1 to G3) single $Terc^{-/-}$ as well as double mutant $Terc^{-/-}/PMS2^{-/-}$ primary MEF using the 3T3 cell passage protocol (see Materials and Methods; Todaro and Green 1963). G3 $Terc^{-/-}$ MEF showed a decreased immortalization ability compared with wild-type MEF as indicated by the higher number of passages required to escape the so-called "premature senescence" arrest or "culture shock" and to

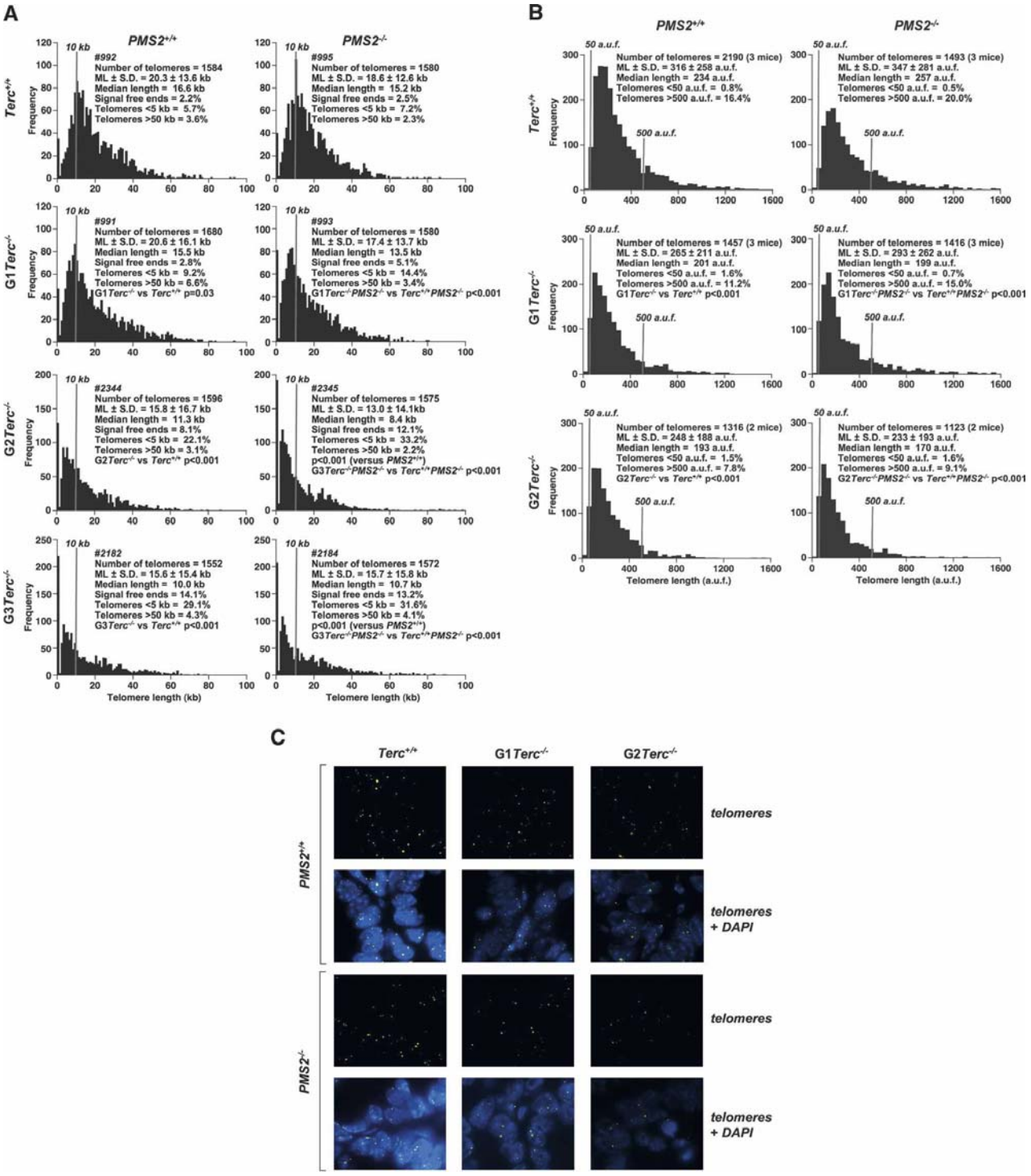

Figure 4. PMS2 deficiency does not rescue telomere shortening in telomerase-deficient mice. (*A*) Telomere fluorescence as determined by Q-FISH in primary MEF (passage 2) of the indicated genotypes. Numbers identify individual MEF cultures. MEFs #991, 992, 993, and 995; #2344 and 2345; as well as #2182 and 2184 were derived from littermate embryos. For each genotype, >1500 telomeres were analyzed by Q-FISH. Mean telomere length in kilobases and standard error are shown. Statistical significance using the Wilcoxon–Mann–Whitney rank sum test is indicated. It is important to note that telomere length values shown in this experiment can be compared between genotypes, as Q-FISH was performed on the same day and in parallel. These values, however, cannot be directly compared with those shown in Supplemental Fig. 6A, as they correspond to an independent Q-FISH experiment with immortalized (passage 35) MEF. (*B*) Q-FISH analysis of small intestine sections in mice of indicated genotypes. Between 60 and 110 nuclei from two to three mice of each genotype were analyzed. Statistical significance using the Wilcoxon–Mann–Whitney rank sum test is indicated. (*C*) Representative images of telomere fluorescence (telomeres), and combined telomere fluorescence and DAPI staining on small intestine sections as determined by Q-FISH.

achieve exponential growth in vitro (Supplemental Fig. 6A,B). In contrast, double mutant G2 and G3 *Terc*$^{-/-}$/*PMS2*$^{-/-}$ MEF were able to immortalize at lower passages compared with the corresponding single mutant Terc-deficient controls, reflecting on their higher proliferative capacity (Supplemental Fig. 6B). Importantly, the higher immortalization ability of *Terc*$^{-/-}$/*PMS2*$^{-/-}$ MEF compared with the single *Terc*$^{-/-}$ MEF was independent of telomere length, which was comparable in immortalized (passage 35) single and double mutant MEF (Supplemental Fig. 6A; histograms reflect individual MEF cultures).

Effects of PMS2 deficiency on telomere dysfunction in late-generation Terc-deficient mice

To address the possible involvement of PMS2 on telomere dysfunction, we first quantified the frequency of cells showing γ-H2AX foci in small intestine sections from late-generation (G2) single *Terc*$^{-/-}$ and double *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice. γ-H2AX foci have been previously described to mark double-strand breaks as well as critically short and dysfunctional telomeres (Modesti and Kanaar 2002; d'Adda di Fagagna et al. 2003). As shown in Figure 5, A and B, increasing generations of *Terc*$^{-/-}$ mice showed progressively higher numbers of small intestine cells with γ-H2AX foci compared with wild-type controls, in agreement with progressively shorter telomeres in these cells. Importantly, the percentage of cells with γ-H2AX foci was not significantly rescued by PMS2 deficiency in late-generation *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice (Fig. 5A,B), suggesting that mutant *Terc*$^{-/-}$/*PMS2*$^{-/-}$ cells bear a similarly high load of DNA damage and telomere dysfunction to that of the single mutant *Terc*$^{-/-}$ controls.

To further study the possible involvement of PMS2 in the generation of chromosomal instability triggered by dysfunctional telomeres, we performed cytogenetic analysis of primary (passage 3) MEF derived from G1 to G3 single *Terc*$^{-/-}$ and double mutant *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice using telomere Q-FISH on metaphase spreads (see Materials and Methods). Again, in agreement with progressive telomere shortening associated with telomerase deficiency, we detected increased frequencies of chromosome ends lacking the TTAGGG signal ("signal-free" ends) in G1–G3 *Terc*$^{-/-}$ MEF ($P < 0.001$ for all comparisons; Supplemental Fig. 7A), which were further increased in the corresponding double mutant *Terc*$^{-/-}$/*PMS2*$^{-/-}$ MEF ($P < 0.001$ when comparing G2–G3 *Terc*$^{-/-}$ MEF to G2–G3 *Terc*$^{-/-}$/*PMS2*$^{-/-}$ MEF; Supplemental Fig. 7A). Accordingly, end-to-end fusions lacking TTAGGG signals at the fusion point were increased both in late-generation G3 *Terc*$^{-/-}$ MEF and G3 *Terc*$^{-/-}$/*PMS2*$^{-/-}$ MEF ($P < 0.001$; Supplemental Fig. 7A). Of note, abrogation of only PMS2 also resulted in slightly increased end-to-end fusions ($P = 0.013$; Supplemental Fig. 7A); however, these fusions are unlikely to result from telomere shortening, as *PMS2*-deficient cells show normal telomere length (Fig. 4A,B). *PMS2*-deficient cells also showed increased frequencies of breaks and fragments (Supplemental Fig. 7A), as well as of a number of complex aberrations including bivalent recombination figures and chromatid cross-links, complex chromosome fusions, and minichromosomes (Supplemental Fig. 7B), probably reflecting on the known roles of PMS2 in MMR and mitotic recombination (Jiricny 2006). Some of these aberrations, such as breaks and fragments, bivalent recombination figures, and complex aberrations, were also increased in late-generation G3 *Terc*$^{-/-}$ mice ($P < 0.05$ for all comparisons; Supplemental Fig. 7A,B), suggesting that they may be also triggered by short dysfunctional telomeres. Interestingly, the increased bivalent recombination figures and chromatid cross-links detected in late-generation G3 *Terc*$^{-/-}$ mice were rescued in the absence of PMS2, suggesting a role for MMR in the origin of these lesions (Supplemental Fig. 7B). In turn, the increased number of complex chromosome aberrations and fusions, as well as increased minichromosomes found in single *PMS2*-deficient cells, were partially rescued by *Terc*-deficiency and short telomeres (Supplemental Fig. 7B). This partial rescue of complex chromosomal aberrations cannot be explained by a lower proliferative potential of double mutant *Terc*$^{-/-}$/*PMS2*$^{-/-}$ MEF compared with the single *PMS2*$^{-/-}$ controls, as *Terc*$^{-/-}$/*PMS2*$^{-/-}$

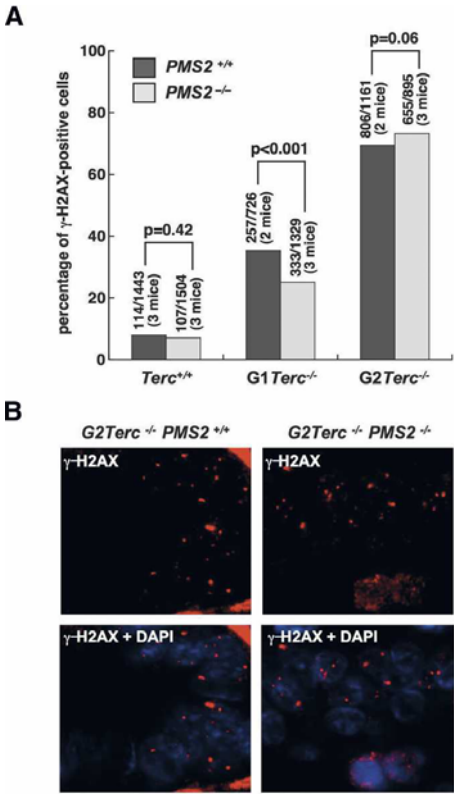

Figure 5. Similar numbers of γH2AX-positive cells in *Terc*$^{-/-}$/*PMS2*$^{+/+}$ and *Terc*$^{-/-}$/*PMS2*$^{-/-}$ small intestine sections. (*A*) Quantification of the percentage of nuclei containing γH2AX foci in small intestine sections from mice of the indicated genotypes. Between 700 and 1500 nuclei were examined in a total of two to three mice per genotype. Statistical calculations using the χ^2 test are shown. (*B*) Representative images of γH2AX-positive cells in small intestine sections from G2 *Terc*$^{-/-}$/*PMS2*$^{+/+}$ and G2 *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice are shown.

MEF have increased proliferative rates compared with the single controls (Supplemental Fig. 6B). Finally, the partial rescue of PMS2-associated complex chromosomal aberrations and minichromosomes in double mutant *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice may be related, at least in part, to the decreased tumorigenesis shown by these mice compared with the single *PMS2*-deficient controls (see Fig. 2A).

All together, these results indicate that mice and cells doubly deficient for PMS2 and telomerase show severe telomere dysfunction as indicated by similar frequencies of γ-H2AX foci, as well as by similar frequencies of end-to-end fusions and signal-free ends compared with single Terc-deficient controls, suggesting that PMS2 deficiency does not rescue survival or proliferation of *Terc*-deficient mice by lowering DNA damage associated with telomere dysfunction.

Sister telomere recombination frequencies in double mutant Terc$^{-/-}$/PMS2$^{-/-}$ *mice*

The fact that MMR deficiency was able to rescue proliferation defects in telomerase-deficient yeast strains was interpreted according to the known role of MMR in inhibiting recombination between imperfect repeats (Modrich and Lahue 1996), which in turn could be favoring activation of ALT pathways for telomere maintenance (Rizki and Lundblad 2001). Similarly, the fact that double mutant *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice show an improved survival compared with single telomerase-deficient mice may be suggesting a similar role for mammalian PMS2 in repressing telomere recombination. In order to directly test this, we determined the frequency of telomeric sister chromatid exchange events (T-SCE) in primary MEF from different genotype mice using two-color chromosome orientation FISH (CO-FISH) (Bailey et al. 2004; Gonzalo et al. 2006). Of note, CO-FISH allows detection of recombination events between sister telomeres but not between subtelomeric repeats or telomeres at different chromosomes. The strand-specific nature of the CO-FISH typically yields two telomeric signals of each color (red: lagging; green: leading) per chromosome in the absence of recombination events (Fig. 6). A sister chromatid exchange within telomeric DNA (T-SCE) leads to the mixture of red and green fluorescence (Fig. 6). We counted as positive T-SCE events only those that were detected both with the leading and the lagging telomere

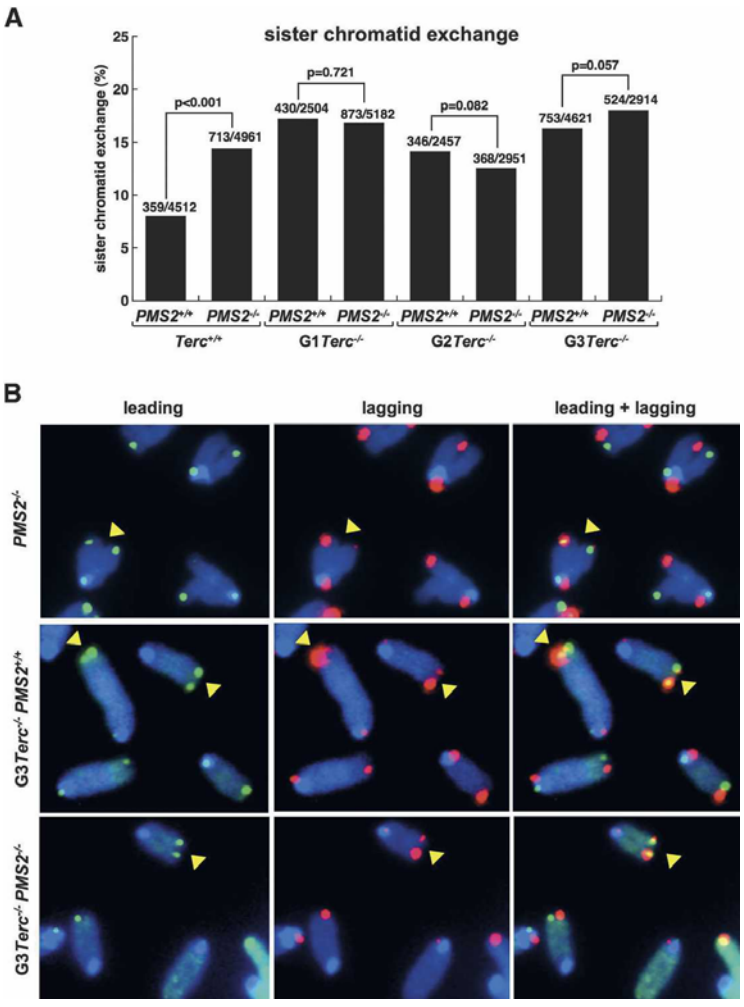

Figure 6. Telomere recombination in *Terc*$^{-/-}$/*PMS2*$^{-/-}$ MEF is equivalent to that of single mutant *Terc*$^{-/-}$ MEF. (*A*) Quantification of T-SCE frequencies in the indicated genotypes. The number of T-SCE events out of the total number of chromosomes analyzed is indicated on *top* of each bar. Statistical calculations using the χ^2 test are shown. (*B*) Representative CO-FISH images of metaphases hybridized with probes against the leading (green fluorescence) and lagging (red fluorescence) telomere. (Yellow arrows) T-SCE events. A T-SCE was considered positive when it was observed with both the leading and lagging strand probes and involved an unequal exchange of telomere signal.

probes, as well as involving an unequal exchange of telomeric signal (Fig. 6). Interestingly, PMS2 deficiency led to significantly increased T-SCE frequencies compared with wild-type cells ($P < 0.001$; Fig. 6A), suggesting that PMS2 may be repressing these events at telomeres, in agreement with the known role of MMR in repressing mitotic recombination. T-SCE events were also augmented in different-generation *Terc*$^{-/-}$ mice compared with the wild-type controls (Fig. 6A; $P < 0.001$ for all comparisons between generations; see "statistics" in Supplemental Material), in agreement with previous reports showing increased T-SCE in the absence of telomerase (Benetti et al. 2007; Blanco et al. 2007). Simultaneous absence of both PMS2 and Terc, however, did not significantly increase T-SCE frequencies in double mutant *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice compared with the single controls ($P > 0.05$ for all comparisons; Fig. 6A), suggesting that increased telomere recombination between sister telomeres is unlikely to be responsible for the rescue of survival in these mice. These results are also in agreement with the fact that *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice did not show elongated telomeres or a rescue in end-to-end fusions compared with the single mutant controls, which would have been expected from increased telomere recombination frequencies (Fig. 4). We cannot rule out, however, that PMS2 deficiency may be having an effect on the frequencies of recombination events between subtelomeres or telomeres on different chromosomes, although this effect is not sufficient to rescue telomere shortening and the elevated frequencies of end-to-end fusions and critically short telomeres in *PMS2*/*Terc*-deficient cells.

Microsatellite instability in Terc$^{-/-}$/PMS2$^{-/-}$ *mice*

PMS2 deficiency results in increased microsatellite instability as the result of the defective MMR pathway (Prolla et al. 1998). Since microsatellite instability has been proposed to be one of the causes of increased cancer in these mice, we addressed here whether simultaneous PMS2 and telomerase deficiencies affected the frequency of microsatellite instability in *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice compared with the single mutant controls, which could explain the lower cancer incidence of double mutant *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice compared with the single *PMS2*$^{-/-}$ controls. To this end, we measured microsatellite instability in tail DNA from the different mouse genotypes (Supplemental Fig. 8). In particular, we determined microsatellite instability using the CA repeat loci D6Mit59 previously shown to be unstable in PMS2-deficient cells (Baker et al. 1995), as well as the D1Mit62 stable locus as a control (see Materials and Methods). As shown in Supplemental Figure 8, no alterations were found at the stable locus in any of the genotypes. In contrast, *PMS2*$^{-/-}$ deficient cells showed 71% novel alleles at the D6Mit59 locus compared with only 36% in the wild-type controls (Supplemental Fig. 8). Increasing generations of *Terc*-deficient mice also resulted in a progressive increase of novel alleles at the D6Mit59 locus, going from 44% in G1, 62% in G2, and 100% in G3 *Terc*$^{-/-}$ mice, which opens the intriguing possibility that telomere shortening may lead to microsatellite instability (Supplemental Fig. 8). Of note, simultaneous Terc and PMS2 deficiencies in double mutant *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice resulted in similarly elevated frequencies of novel D6Mit59 alleles (Supplemental Fig. 8), suggesting a similar microsatellite instability in these double mutant mice compared with the single mutant *PMS2*$^{-/-}$ controls.

PMS2 deficiency results in an attenuated p21 induction in response to short telomeres

A role for MMR proteins in DNA damage signaling, which in turn could affect tumorigenesis, has also been proposed; in particular, MMR proteins have been directly involved in signaling DNA damage through the p53 pathway (Duckett et al. 1999; Peters et al. 2003; Luo et al. 2004). In addition, PMS2 has been identified as a direct target for p53 (Chen and Sadowski 2005). To address whether the rescue of *Terc*$^{-/-}$ mice survival mediated by PMS2 deficiency could be due to a role for PMS2 in response to telomere dysfunction, and given the similarity of this phenotype with that observed in the absence of p21 (Choudhury et al. 2007), we first determined p21 levels by immunohistochemistry directly on small intestine sections from different genotype mice (see Materials and Methods). Increasing generations of single *Terc*$^{-/-}$ mice showed elevated amounts of p21 in the GI tract (Fig. 7A,B), in agreement with decreased proliferation rates in these mice (Fig. 3A,B). Interestingly, PMS2 deficiency dramatically decreased p21 accumulation in the small intestine of different-generation *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice (Fig. 7A,B), in agreement with the concomitant rescue in proliferation defects (Fig. 3A,B). These results demonstrate an attenuated p21 induction in vivo in response to telomere shortening in the absence of PMS2.

Next, we confirmed these results by measuring p21 protein amounts by Western blot in different-genotype MEF, both in basal conditions, as well as after ionizing radiation (Supplemental Fig. 9A,B). Again, we detected increased p21 protein levels in *G2 Terc*$^{-/-}$ mice compared with wild-type controls, while single PMS2-deficient cells showed normal p21 levels (Supplemental Fig. 9A,B). Interestingly, this accumulation of p21 protein associated with short telomeres was largely abrogated by the simultaneous absence of PMS2 in G2 *Terc*$^{-/-}$/*PMS2*$^{-/-}$ MEF (Supplemental Fig. 9A,B). As a positive control for p21 induction in response to DNA damage, we show increased p21 protein levels in response to 10 Gy of γ-irradiation (Supplemental Fig. 9A). Quantification of p53 levels by immunofluorescence in the GI tract also showed elevated p53 protein levels in G2 *Terc*$^{-/-}$ mice compared with the *Terc*$^{+/+}$ controls, which were largely rescued in the absence of PMS2 ($P < 0.001$; Supplemental Fig. 9C), in agreement with the fact that p53 is one of the key regulators of p21.

All together, these results indicate an attenuated p21 induction in response to short telomeres in the absence of PMS2, similarly to that recently shown for p21 deficiency in double mutant *Terc*$^{-/-}$/*p21*$^{-/-}$ mice

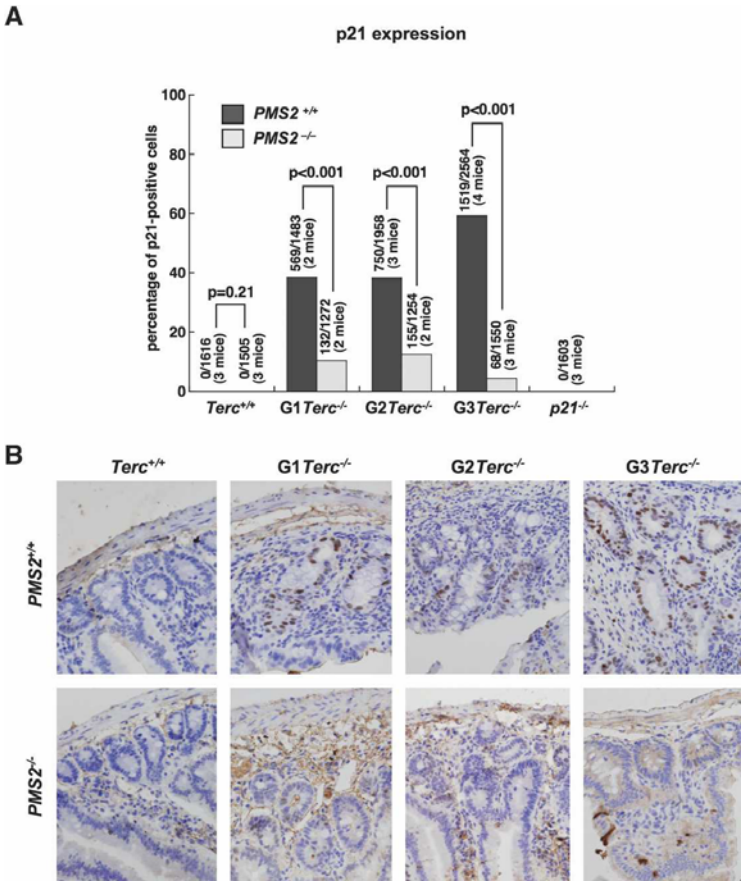

Figure 7. A role for PMS2 in p21 induction in reponse to telomeric dysfunction. (*A*) Quantification of p21 levels by immunohistochemistry in small intestine sections from mice of the indicated genotypes. Statistical comparisons using the χ^2 test are also shown. More than 1200 nuclei from a total of two to four mice per genotype were used for the analysis. (*B*) Representative images showing the dramatic decrease of p21-positive cells in $Terc^{-/-}/PMS2^{-/-}$ small intestine sections compared with the single $Terc^{-/-}$ controls.

(Choudhury et al. 2007). Finally, this attenuated p21 induction in the absence of PMS2 is not likely to be due to a significantly decreased DNA damage in the absence of PMS2 as indicated both by the similarly elevated frequencies of chromosomal aberrations (end-to-end fusions and signal free ends in Supplemental Fig. 7A) and of γH2AX-positive cells (Fig. 5) in single and double mutant $Terc^{-/-}/PMS2^{-/-}$ mice, although we cannot rule out that the decreased number of both breaks and fragments and bivalent recombination figures associated with PMS2 deficiency could be having an effect on p21 induction. All together, these results are in agreement with a role for PMS2 in mediating the cellular response to short and dysfunctional telomeres by attenuating p21 induction.

Discussion

Here, we studied for the first time in a mammalian in vivo model the interaction between telomere function and MMR by generating mice doubly mutant for telomerase and PMS2. We found that telomerase deficiency rescues survival of single PMS2-deficient mice coincidental with decreased tumorigenesis in these mice, extending the observation that short telomeres act as potent tumor suppressors to carcinogenesis driven by defects in MMR. These findings suggest that telomerase inhibitors would be effective in the treatment of tumors bearing PMS2 mutations and microsatellite instability. Secondly, we show that, in an analogous manner to that previously described for telomerase-deficient yeast strains, PMS2 deficiency rescues survival as well as proliferative defects in telomerase-deficient mice with dysfunctional telomeres. We further demonstrate that this rescue occurs in the absence of telomere length changes and without significantly decreasing the DNA damage load associated with severe telomere dysfunction, as indicated by similar frequencies of end-to-end fusions and signal-free ends, as well as of γH2AX-positive cells in vivo. Similarly, in agreement with short telomeres and severe telomere dysfunction in these mice, we do not find elevated sister telomere recombination frequencies in mice doubly deficient for telomerase and PMS2 that could explain the rescue in survival. This is unlike the previously proposed role for MMR genes in controlling telomere recombination and telomere length at yeast telomeres, which in turn could rescue proliferation defects in telomerase-deficient yeast strains. Instead, the data presented here suggest a novel role for PMS2 in the cellular response to short and dysfunctional telomeres. In particular, PMS2 deficiency results in an attenuated p21 induction in response to dysfunctional telomeres both in vivo and in vitro. This attenuated p21 induction coincides with a rescue of proliferation but not of apoptosis in $Terc^{-/-}/PMS2^{-/-}$ mice compared with the single

Terc$^{-/-}$ controls, in the absence of increased tumorigenesis. This rescue of proliferation defects could explain the increased survival of *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice compared with the single *Terc*$^{-/-}$ controls. This situation is analogous to that recently described for mice doubly deficient for Terc and p21. In particular, *Terc*$^{-/-}$/*p21*$^{-/-}$ mice show a rescue of organismal survival independently of telomere length and telomere dysfunction, which is not accompanied by increased tumorigenesis (Choudhury et al. 2007). Furthermore, similarly to *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice, *Terc*$^{-/-}$/*p21*$^{-/-}$ mice show a rescue of proliferative defects but not of apoptosis (Choudhury et al. 2007). These results suggest that PMS2 and p21 may be in the same genetic pathway that has evolved to signal cell cycle arrest associated with telomere dysfunction. Finally, these results also suggest that cell cycle arrest may be dominant over apoptosis in eliciting age-related pathologies in the telomerase-deficient mouse model. It is important to point out that, in marked contrast to p21 and PMS2 deficiencies, p53 deficiency does not rescue survival of telomerase-deficient mice in spite of the fact p53 deficiency rescues both proliferative defects and apoptosis associated with telomere dysfunction (Chin et al. 1999). A possible explanation for this discrepancy is the fact that p53 deficiency leads to rapid tumor development, which is not impaired by telomerase deficiency (Chin et al. 1999). Instead, telomere dysfunction accelerates p53-induced carcinogenesis by promoting chromosomal instability (Artandi et al. 2000). In this regard, it is interesting to note that, in contrast to PMS2 and p21 deficiencies, p53 deficiency rescues apoptosis associated with telomere dysfunction, suggesting that this differential effect on apoptosis may be of relevance to explain the different carcinogenesis outputs.

Materials and methods

Generation and genotyping of mice

To generate *PMS2*$^{-/-}$/*Terc*$^{-/-}$ mice, *PMS2*$^{-/-}$ females were crossed with *Terc*$^{-/-}$ males (Baker et al. 1995; Blasco et al. 1997). Genotyping was performed as described (Baker et al. 1995; Blasco et al. 1997).

Mice and primary mouse cells

Mouse colonies of successive generations (G1 to G3) of single *Terc*$^{-/-}$ and of double *Terc*$^{-/-}$/*PMS2*$^{-/-}$ mice were generated in a C57BL6 background and maintained at the CNIO under specific-pathogen-free conditions in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations.

Primary MEFs were prepared from day 13.5 embryos, as previously described (Blasco et al. 1997). Serial 3T3 cultivation was done as described (Todaro and Green 1963). Briefly, 10^6 cells were plated on 10-cm diameter dishes; 3 d later the total number of cells in the dish was counted, and 10^6 cells were replated again. The increase in population doubling level (ΔPDL) was calculated according to the formula PDL = $\log(n_f/n_0)/\log 2$, where n_0 is the initial number of cells and n_f is the final number of cells. For calculation of PD at which the culture escaped senescence, we considered a valid change in the slope of the curve when, after the inflection point, the following six data points (passage numbers) increased the number of PDLs by at least two.

Telomere length analysis by quantitative FISH

Exponentially growing primary MEFs were incubated with 0.1 µg/mL colcemide (Gibco) for 4 h at 37°C and then fixed in methanol:acetic acid (3:1). Quantitative FISH was performed as described (Herrera et al. 1999; Samper et al. 2000). To correct for lamp intensity and alignment, images from fluorospheres (fluorescent beads; Molecular Probes) were analyzed using the TFL-Telo software (gift from Dr. Peter Lansdorp). Telomere fluorescence values were extrapolated from the telomere fluorescence of lymphoma cell lines LY-R (R cells) and LY-S (S cells) with known telomere lengths of 80 and 10 kb, respectively. There was a linear correlation ($r^2 = 0.999$) between the fluorescence intensity of the R and S telomeres. We recorded the images using a COHU CCK camera on a fluorescence microscope (DMRb, Leica). A mercury vapor lamp (CS 100 W-2; Philips) was used as a source. We captured the images using the Leica Q-FISH software in a linear acquisition mode to prevent oversaturation of fluorescence intensity. We used the TFL-Telo software (Zijlmans et al. 1997) to quantify the fluorescence intensity of telomeres from at least 10 metaphases for each data point.

For Q-FISH analysis on small intestine sections, deparaffinated sections were hybridized with a PNA-telomeric probe, and telomere fluorescence was determined as described (González-Suárez et al. 2000; Muñoz et al. 2005). More than 60 nuclei from each mouse and condition were captured at 100× magnification by using a Leica CTR MIC microscope and a COHU High Performance CCD camera. Telomere fluorescence was integrated using spot IOD analysis in the TFL-TELO program (kindly provided by Dr. Landsdorp, Vancouver, Canada) (Zijlmans et al. 1997).

As median length, we refer to the telomere length value, which divides the entire population of telomeres in two equal parts, i.e., the value at which 50% of the telomeres are shorter than the median telomere length value and 50% are longer.

TRF-based telomere length analysis of MEF

Primary MEF of the indicated genotypes were included in agarose plugs following instructions provided by the manufacturer (Bio-Rad), and TRF analysis was performed as previously described (Blasco et al. 1997).

Cytogenetic analysis using telomere Q-FISH on metaphases

For analysis of chromosomal aberrations, at least 50 metaphases per genotype were analyzed by superimposing the telomere image on the DAPI image using the TFL-telo software.

Telomere recombination measurements using chromosome orientation FISH (CO-FISH)

Exponentially growing primary MEFs were subcultured in the presence of 5′-bromo-2′-deoxyuridine (BrdU; Sigma) at a final concentration of 1×10^{-5} M, and then allowed to replicate their DNA once for 24 h at 37°C. Colcemide was added at a concentration of 1 µg/mL during the last 4 h. Cells were then recovered and metaphases prepared as described (Samper et al. 2000). CO-FISH was performed as described (Bailey et al. 2004; Gonzalo et al. 2006) using first a $(TTAGGG)_7$ probe labeled with Cy3 and then a second $(CCCTAA)_7$ probe labeled with Rhodamine

Green (Applied Biosystems). Metaphase spreads were captured on a Leitz Leica DMRB fluorescence microscope.

Histopathology and immunohistochemistry

Small intestine sections were fixed in 10% buffered formalin, embedded in paraffin wax, and sectioned at 2–3 µm. Immunohistochemistry was performed on d-paraffinated intestine sections processed with 10 mM sodium citrate (pH 6.5) cooked under pressure for 2 min. Slides were washed in water, washed in Buffer TBS Tween 20 0.5%, blocked with peroxidase, washed with TBS Tween 20 0.5% again, and blocked with fetal bovine serum followed by another wash. Then the slides were incubated with the primary antibodies: rabbit monoclonal to Ki-67 antibody (prediluted, SP6, Master Diagnostica), active caspase 3 at 1:200 (R&D Systems), or p21 at 1:15 (C-19-G, Santa Cruz). Slides were then incubated with secondary antibodies conjugated with peroxidase from DAKO, goat anti-rabbit (1:50) in the case of Ki-67 and active caspase 3 and rabbit anti-goat in the case of p21. For signal development, DAB (DAKO) was used as a substrate. Sections were lightly counterstained with hematoxylin and analyzed by light microscopy.

Phosphorylated H2AX foci (γ-H2AX) were detected using a mouse monoclonal anti-phospho-histone H2AX antibody (1:500; Upstate Biotechnology). After incubation with Cy3-goat anti-mouse antibody (1:400; Jackson ImmunoResearch Laboratories, Inc.) for 30 min at room temperature, slides were mounted in Vectashield with 4′,6-diamino-2 phenylindole (DAPI). Images were obtained using a fluorescence microscope (Leica DMRB).

Intestinal lesions were classified as mild, moderate, and severe lesions according to the pathological findings. Mild lesions were characterized by mild muscular atrophy of the small and/or large intestine. Moderate lesions showed a partial atrophy of the small and/or large intestine. Severe lesions were those characterized by (1) severe muscular atrophy, (2) severe inflammation and/or ulceration, enteritis/peritonitis, or (3) by intestinal atrophy (independent of its grade) in combination with other pathologies indicative of severe intestinal dysfunction (inflammation and/or ulceration, enteritis peritonitis).

Western blots

Whole-cell extracts were prepared from nonirradiated and irradiated primary MEF as described (Blanco et al. 2007). Protein concentration was determined using the Bio-Rad DC Protein Assay. Twenty-five micrograms of each extract was separated in 4%–20% gradient SDS-polyacrylamide gels by electrophoresis. After transfer, the membranes were incubated with an anti-p21 C-19 polyclonal antibody (1:250; Santa Cruz Biotechnology) and anti-β-actin monoclonal (1:10.000; Sigma). Antibody binding was detected after incubation with a secondary antibody coupled to horseradish peroxidase using enhanced chemiluminescence.

γ-Irradiation of primary MEF

A total of 3×10^5 MEFs were seeded in 60-mm plates and incubated overnight at 37°C. Then, MEFs were treated with 10 Gy by using a ^{137}Cs source (MARK 1–30 irradiator; Shepherd & Associates) at a rate of 2.11 Gy/min. After removing the medium, cells were washed and fresh medium was added. To analyze the effect of γ-irradiation on protein expression, cells were incubated for 3–18 h at 37°C and then recovered and frozen for further analysis by Western blot.

Quantification of p53 expression by immunofluorescence

High-throughput quantitative image analysis of p53 expression levels in intestine sections was performed on fluorescence microscope images using the Metamorph platform (version 6.3r6; Molecular Devices). The DAPI image was used to define the nuclear area and the Cy3 image for quantification of p53 fluorescence. The DAPI images were signal-intensity thresholded, segmented, and converted to a 1-bit binary image. The binary DAPI mask was applied to the matching Cy3 to obtain a combined image with p53 fluorescence information for each nucleus. Cy3 fluorescence intensity (p53 fluorescence) was measured as "average gray values" units (arbitrary units of fluorescence). Finally, p53 fluorescence values for each histological region were exported to Microsoft Excel, and the frequency histograms were generated. p53-deficient mice were used as a negative control for p53 expression.

Microsatellite instability (MSI)

Microsatellite instability in tail DNA was assayed by PCR amplification (Baker et al. 1995) with primers for D6Mit59 (forward primer: 5′-TGTGCCATGACAGAGGGAAA-3′ and reverse primer: 5′-GAAGAAGCTGCCATCCTTTGTAATAA-3′) and D1Mit62, the control primer set (forward primer: 5′-CCTGA GTTCAGTTATCAGCGC-3′ and reverse primer: 5′-GAGAC CAGAAGAGCGTGTCC-3′). Then the products were loaded onto a 3% high-resolution agarose gel to determine allelic variants (MetaPhor Agarose, Cambrex).

Statistical analysis

A log rank test was used to calculate statistical differences in survival and median survival of the different mouse cohorts.

A *t*-student test was used to calculate the statistical significance of the observed differences in mean lifespan.

The Wilcoxon–Mann–Whitney rank sum test was used for statistical comparisons of the mean telomere length in MEFs and small intestine sections.

A χ^2 test was used to calculate statistical differences in pathologies, Q-FISH analysis of MEF (signal free ends, telomeres <5 kb), chromosomal aberrations, γH2AX, sister chromatid exchange, apoptosis, proliferation, cell loss, p21, and p53 expression.

Acknowledgments

We thank E. Santos and R. Serrano for mouse care and genotyping. I.S.-C. is a predoctoral fellow founded by the Spanish Ministry of Education and Culture (MEC). P.M. is a Ramon y Cajal senior scientist. M.A. Blasco's laboratory is funded by the MEC (SAF2001-1869, GEN2001-4856-C13-08), by the CAM (08.1/0054/01), European Union (TELOSENS FIGH-CT-2002-00217, INTACT LSHC-CT-2003-506803, ZINCAGE FOOD-CT-2003-506850, RISC-RAD FI6R-CT-2003-508842, MOL CANCER MED LSHC-CT-2004-502943), and the Josef Steiner Award 2003.

References

Artandi, S.E., Chang, S., Lee, S.L., Alson, S., Gottlieb, G.J., Chin, L., and DePinho, R.A. 2000. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature* **406:** 641–645.

Bailey, S.M., Brenneman, M.A., and Goodwin, E.H. 2004. Fre-

quent recombination in telomeric DNA may extend the proliferative life of telomerase-negative cells. *Nucleic Acids Res.* **32:** 3743–3751.

Baker, S.M., Bronner, C.E., Zhang, L., Plug, A.W., Robatzek, M., Warren, G., Elliott, E.A., Yu, J., Ashley, T., Arnheim, N., et al. 1995. Male mice defective in the DNA mismatch repair gene PMS2 exhibit abnormal chromosome synapsis in meiosis. *Cell* **82:** 309–319.

Bechter, O.E., Zou, Y., Walker, W., Wright, W.E., and Shay, J.W. 2004. Telomeric recombination in mismatch repair deficient human colon cancer cells and telomerase inhibition. *Cancer Res* **64:** 3444–3451.

Benetti, R., Garcia-Cao, M., and Blasco, M.A. 2007. Telomere length regulates the epigenetic status of mammalian telomeres and subtelomeres. *Nat. Genet.* **39:** 243–250.

Blanco, R., Muñoz, P., Klatt, P., Flores, J.M., and Blasco, M.A. 2007. Telomerase abrogation dramatically accelerates TRF2-induced epithelial carcinogenesis. *Genes & Dev.* **21:** 206–220.

Blasco, M.A. 2005. Telomeres and human disease: cancer, ageing and beyond. *Nat. Rev. Genet.* **6:** 611–622.

Blasco, M.A. and Hahn, W.C. 2003. Evolving views of telomerase and cancer. *Trends Cell Biol.* **13:** 289–294.

Blasco, M.A., Lee, H.-W., Hande, P., Samper, E., Lansdorp, P., DePinho, R., and Greider, C.W. 1997. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* **91:** 25–34.

Bronner, C.E., Baker, S.M., Morrison, P.T., Warren, G., Smith, L.G., Lescoe, M.K., Kane, M., Earabino, C., Lipford, J., Lindblom, A., et al. 1994. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary nonpolyposis colon cancer. *Nature* **368:** 258–261.

Brown, K.D., Rathi, A., Kamath, R., Beardsley, D.I., Zhan, Q., Mannino, J.L., and Baskaran, R. 2003. The mismatch repair system is required for S-phase checkpoint activation. *Nat. Genet.* **33:** 80–84.

Chan, S.W.-L. and Blackburn, E.H. 2002. New ways not to make ends meet: Telomerase, DNA damage proteins and heterochromatin. *Oncogene* **21:** 553–563.

Chen, J. and Sadowski, I. 2005. Identification of the mismatch repair genes PMS2 and MLH1 as p53 target genes by using serial analysis of binding elements. *Proc. Natl. Acad. Sci.* **102:** 4813–4818.

Chin, L., Artandi, S.E., Shen, Q., Tam, A., Lee, S.L., Gottlieb, G.J., Greider, C.W., and DePinho, R.A. 1999. p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. *Cell* **97:** 527–538.

Choudhury, A.R., Ju, Z., Djojosubroto, M.W., Schienke, A., Lechel, A., Schaetzlein, S., Jiang, H., Stepczynska, A., Wang, C., Buer, J., et al. 2007. Cdkn1a deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation. *Nat. Genet.* **39:** 99–105.

d'Aadda di Fagagna, F., Reaper, P.M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N.P., and Jackson, S.P. 2003. DNA damage checkpoint response in telomere-initiated senescence. *Nature* **426:** 194–198.

de Lange, T. 2005. Shelterin: The protein complex that shapes and safeguards human telomeres. *Genes & Dev.* **19:** 2100–2110.

de Wind, N., Dekker, M., Berns, A., Radman, M., and te Riele, H. 1995. Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell* **82:** 321–330.

Duckett, D.R., Bronstein, S.M., Taya, Y., and Modrich, P. 1999. hMutSα- and hMutLα-dependent phosphorylation of p53 in response to DNA methylator damage. *Proc. Natl. Acad. Sci.* **96:** 12384–12388.

Dunham, M.A., Neumann, A.A., Fasching, C.L., and Reddel, R.R. 2000. Telomere maintenance by recombination in human cells. *Nat. Genet.* **26:** 447–450.

Espejel, S. and Blasco, M.A. 2002. Identification of telomere-dependent "senescence-like" arrest in mouse embryonic fibroblasts. *Exp. Cell Res.* **276:** 242–248.

Fishel, R., Lescoe, M.K., Rao, M.R., Copeland, N.G., Jenkins, N.A., Garber, J., Kane, M., and Kolodner, R. 1993. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell* **77:** 167.

González-Suárez, E., Samper, E., Flores, J.M., and Blasco, M.A. 2000. Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis. *Nat. Genet.* **26:** 114–117.

Gonzalo, S., Jaco, I., Fraga, M.F., Chen, T., Li, E., Esteller, M., and Blasco, M.A. 2006. DNA methytransferases control telomere length and telomere recombination in mammalian cells. *Nat. Cell Biol.* **8:** 416–424.

Goytisolo, F.A. and Blasco, M.A. 2002. Many ways to telomere dysfunction: In vivo studies using mouse models. *Oncogene* **21:** 584–591.

Greenberg, R.A., Chin, L., Femino, A., Lee, K.H., Gottlieb, G.J., Singer, R.H., Greider, C.W., and DePinho, R.A. 1999. Short dysfunctional telomeres impair tumorigenesis in the INK4a(delta2/3) cancer-prone mouse. *Cell* **97:** 515–525.

Herrera, E., Samper, E., Martin-Caballero, J., Flores, J.M., Lee, H.W., and Blasco, M.A. 1999. Disease states associated to telomerase deficiency appear earlier in mice with short telomeres. *EMBO J.* **18:** 2950–2960.

Jiricny, J. 2006. The multifaceted mismatch-repair system. *Nat. Rev. Mol. Cell Biol.* **7:** 335–346.

Leach, F.S., Nicolaides, N.C., Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L.A., Nystrom-Lahti, M., et al. 1993. Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell* **75:** 1215–1225.

Lee, H.-W., Blasco, M.A., Gottlieb, G.J., Greider, C.W., and DePinho, R.A. 1998. Essential role of mouse telomerase in highly proliferative organs. *Nature* **392:** 569–574.

Luo, Y., Lin, F.T., and Lin, W.C. 2004. ATM-mediated stabilization of hMutL DNA mismatch repair proteins augments p53 activation during DNA damage. *Mol. Cell. Biol.* **24:** 6430–6444.

Modesti, M. and Kanaar, R. 2002. DNA repair: Spot(light)s on chromatin. *Curr. Biol.* **11:** 229–232.

Modrich, P. 1994. Mismatch repair, genetic stability, and cancer. *Science* **266:** 1959–1960.

Modrich, P. and Lahue, R. 1996. Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu. Rev. Biochem.* **65:** 101–133.

Muñoz, P., Blanco, R., Flores, J.M., and Blasco, M.A. 2005. XPF nuclease-dependent telomere loss and increased DNA damage in mice overexpressing TRF2 result in premature aging and cancer. *Nat. Genet.* **10:** 1063–1071.

Muntoni, A. and Reddel, R.R. 2005. The first molecular details of ALT in human tumor cells. *Hum. Mol. Genet.* **14:** 191–196.

Nicolaides, N.C., Papadopoulos, N., Liu, B., Wei, Y.F., Carter, K.C., Ruben, S.M., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., et al. 1994. Mutations of two PMS homologues in hereditary nonpolyposis colon cancer. *Nature* **371:** 75–80.

Papadopoulos, N., Nicolaides, N.C., Wei, Y.F., Ruben, S.M.,

Carter, K.C., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., et al. 1994. Mutation of a mutL homolog in hereditary colon cancer. *Science* **263:** 1625–1629.

Peters, A.C., Young, L.C., Maeda, T., Tron, V.A., and Andrew, S.E. 2003. Mammalian DNA mismatch repair protects cells from UVB-induced DNA damage by facilitating apoptosis and p53 activation. *DNA Repair (Amst.)* **2:** 427–435.

Prolla, T.A., Baker, S.M., Harris, A.C., Tsao, J.L., Yao, X., Bronner, C.E., Zheng, B., Gordon, M., Reneker, J., Arnheim, N., et al. 1998. Tumour susceptibility and spontaneous mutation in mice deficient in Mlh1, Pms1 and Pms2 DNA mismatch repair. *Nat. Genet.* **18:** 276–279.

Rizki, A. and Lundblad, V. 2001. Defects in mismatch repair promote telomerase-independent proliferation. *Nature* **411:** 647–648.

Samper, E., Goytisolo, F.A., Slijepcevic, P., van Buul, P.P., and Blasco, M.A. 2000. Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and the G-strand overhang. *EMBO Rep.* **1:** 244–252.

Shay, J.W. and Wright, W.E. 2006. Telomerase therapeutics for cancer: Challenges and new directions. *Nat. Rev. Drug Discov.* **5:** 577–584.

Shibata, D., Peinado, M.A., Ionov, Y., Malkhosyan, S., and Perucho, M. 1994. Genomic instability in repeated sequences is an early somatic event in colorectal tumorigenesis that persists after transformation. *Nat. Genet.* **6:** 273–281.

Todaro, G.J. and Green, H. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* **17:** 299–313.

van Steensel, B., Smogorzewska, A., and de Lange, T. 1998. TRF2 protects human telomeres from end-to-end fusions. *Cell* **92:** 401–413.

Zijlmans, J.M., Martens, U.M., Poon, S.S., Raap, A.K., Tanke, H.J., Ward, R.K., and Lansdorp, P.M. 1997. Telomeres in the mouse have large inter-chromosomal variations in the number of T2AG3 repeats. *Proc. Natl. Acad. Sci.* **94:** 7423–7428.

11. DISCUSSION

11.1. Telomerase reverses epidermal hair follicle stem cell defects and loss of long-term survival associated with critically short telomeres

Understanding the regulation of proliferative memory in cells contributes to better knowledge of the mechanisms responsible for cancer and aging. In most somatic cells, the telomeres shorten continuously, thereby controlling the proliferative memory [18, 60]. The consequences of aging related to telomere shortening include multiple organ defects and compromised tissue regeneration capacity, likely due to impaired stem cell function [18]. Using the telomerase deficient mouse model, the relationship between telomere shortening and stem cell biology was investigated in-depth. Interestingly, telomere shortening gave rise to mobilization defects of the hair follicle stem cells out of their niche and, as a result, the natural regeneration process of the hair and the skin was impaired [34]. A consequence of these defects is represented by the premature skin-aging phenotype of telomerase-deficient mice [25, 51, 52]. A potential gene therapy to prevent, or at least improve, age-related diseases caused by critically short telomeres would be the reconstitution of telomerase activity. To study this model, telomerase was re-introduced into late generation telomerase-deficient mice and the epidermal hair follicle stem cell biology, survival, and pathologies were studied.

11.1.1. Reconstitution of telomerase activity and telomere biology

We confirmed that telomerase activity was successfully reconstituted in the heterozygous offspring of crosses between late generation telomerase-deficient mice and “normal” mice heterozygous for telomerase. Their homozygous littermates were telomerase deficient. We analyzed the effect of telomerase re-introduction on telomere length in skin keratinocytes. In 2001, Samper et al. showed that telomere length in splenocytes was prolonged by restoration of telomerase activity. We further confirmed this result in skin keratinocytes in which telomeres were elongated and the percentage of short telomeres was considerably diminished. Chromosomal defects, like end-to-end fusions and signal-free ends, are characteristic of late-generation telomerase-deficient cells [65]. Splenocytes with restored telomerase activity have a decreased frequency of end-to-end fusions compared to splenocytes without telomerase. In another study using similar crosses, Hemann et al. (2001) found out that only half of the chromosomes of the telomerase null offspring were short. They used spectral karyotyping (SKY) analysis to show that chromosomes with critically short telomeres lost preferentially telomere functionality and concluded that the shortest telomeres, not the average telomeres, are responsible for telomere dysfunction and subsequent limitations on cellular survival

[179]. Teixeira et al. (2004) demonstrated that telomerase does not act on every telomere in every cell cycle. At least two distinct telomeric states influence telomerase activity: one allows the association with telomerase and the other one avoids telomerase-mediated telomere extension [180]. These insights are in agreement with our results - the high frequency of critically short telomeres in the late generation telomerase-deficient keratinocytes is dramatically reduced when telomerase is re-introduced. In contrast to the number of short telomeres, the percentage of longest telomeres is considerably increased in telomerase-reconstituted keratinocytes when compared with late generation telomerase-deficient keratinocytes. These findings clearly show that telomere length is complete recovered upon re-introduction of one allele of the terc gene. By chromosomal aberration analysis, we also demonstrated a complete recuperation of signal-free ends and end-to-end fusions in the telomerase-proficient keratinocytes. Consequently, we reasoned that these types of aberrations are due to critical telomere shortening and that stem cell defects seem to be associated with increased chromosomal damage. The amount of chromosomal breaks and fragments was not reduced upon telomerase reintroduction, suggesting that these aberrations are not related to telomere shortening.

11.1.2. Telomerase and epidermal stem cells

Proliferation and migration of stem cells out of their niches is a fundamental process in organ homeostasis. In 1990, Cotsarelis et al. reported that hair follicle stem cells reside in the matrix area of the hair bulb. A characteristic feature of stem cells that has been exploited in several studies is their slow-cycling nature. Therefore, stem cells can be labeled by administration of a label for a defined period, followed by a prolonged label-free chase period. Rapidly dividing cells dilute their label while slowly-dividing cells retain more of the label. These marked stem cells are called label-retaining cells (LRC) and they remain marked for an extended period [181]. TPA (O-tetradecanoylphorbol-13-acetate) is a potent tumor promoter and activates LRC mobilization. Flores et al. (2005) showed that in G1 *Terc*$^{-/-}$ mice, characterized by a slight reduction in telomere length, the mobilization of epidermal stem cells out of the hair follicle niche upon mitogen-induced proliferation was partially inhibited. This inhibitive effect was even more pronounced in G3 *Terc*$^{-/-}$ mice with critically short telomeres. The lower rates of proliferation in the hair follicle stem cell niche and in the adjacent transient-amplifying compartments results in defective hair growth and a stunted hyperplasic response [34]. In experiments with transgenic mice, that constitutively overexpress Tert in the skin (K5-*mTert* mice), an increased epidermal stem cell mobilization was shown upon treatment with proliferation stimulus. This increased stem cell mobilization was accompanied by an increased keratinocyte proliferation, enhanced hair growth and augmented skin hyperplasia [34]. One of our goals was to find out whether elongation of short telomeres, upon restoration of telomerase activity in skin keratinocytes, was enough to rescue epidermal stem cell defects in late generation telomerase-deficient mice. Therefore, we examined epidermal stem cells in the hair follicles of mice with re-introduced telomerase and

compared them to their telomerase-deficient littermates before and after mitogenic activation. We observed a considerable recovery of epidermal hair follicle (HF) stem cell mobilization defects in the mice with restored telomerase activity and as a consequence, a normal restoration of skin functionality. Late generation telomerase-deficient mice exhibit a defective hair follicle (HF) anagen response represented by the same HF length before and after TPA treatment. Conversly, in mice with reconstituted telomerase activity we could detect a significant increase in the hair follicle length after treatment with TPA. Similar results were obtained measuring the inter-follicular epidermis (IFE) thickness, indicating that telomerase restoration is sufficient to recover normal skin homeostasis. The hair-plucking experiment is an alternative way to induce the entry of HFs into anagen phase. We, again, demonstrated that telomerase re-introduction is able to greatly improve the regeneration capacity of the skin, confirming the results obtained by TPA treatment.

Late generation telomerase-deficient cells show chromosomal defects. We measured the amount of apoptosis to examine whether this process removes these defective cells, and normal cells take over their function and propagate,. No detectable apoptosis by active caspase 3 staining was found, suggesting that damaged cells are not eliminated by apoptosis in the skin. Proliferation levels, determined by Ki67 staining, were similar in late generation telomerase-deficient mouse skin compared to skin of mice with recovered telomerase activity. From these results, it seems that stem cell defects occur before severe proliferative defects can be detected. To examine if there are alterations in the keratinocyte differentiation program associated with hyperplasia, markers for keratinocyte differentiation were analyzed. Keratins make up the largest subgroup of intermediate filament (IF) proteins and form a dynamic network of filaments, built from type I/type II heterodimers, in the cytoplasm of epithelial cells. K14 is a skin basal-layer marker and forms a heterotetramer with two K5 molecules. We did not find any changes in K14 expression between the different genotypes. We observed similar results with p63, a member of the p53 family, that plays a pivotal role in ectodermal and epidermal development [182, 183]. No considerable difference was observed in skin of mice with or without telomerase activity.

11.1.3. Proliferation potential of epidermal stem cells in vitro

In 1987, Yann Barrandon and Howard Green described three clonal types of keratinocytes with different capacities for multiplication. Colony-forming human epidermal cells are heterogeneous in their capacity for sustained growth [184]. The clone-forming ability of human keratinocytes in culture can be estimated from cell size: small keratinocytes give rise to clones with high frequency, larger ones do so with lower frequency, and the largest ones, do not at all. Importantly, once a colony is formed, its growth potential is not related to the size of the founding cell [185]. Additionally, it was shown that in clonogenic assays individual colonies derive from single stem cells [184]. We used

clonogenic assays to examine if the proliferation potential in primary keratinocytes with re-introduced telomerase differs from their telomerase deficient littermates. Notably, reestablishment of telomerase activity gave rise to a considerable improvement in the proliferative potential of these cells. This experiment enabled us to determine that the effects of telomere length and telomerase activity on different stem cell compartments are cell-autonomous. This knowledge is very important because it confirms that the effect of restoring telomerase on stem cell behavior is intrinsic to the stem cells and is not controlled by the niche. Thus, therapies based on telomerase reactivation seem to be promising for the treatment of age-related diseases provoked by short telomeres.

11.1.4. Rescue of the small body-size phenotype

Defining characteristics of late generation telomerase-deficient mice are the small body-size, shorter telomeres and a reduced clonogenic potential of epidermal stem cells *in vitro* [34]. We therefore hypothesized that body size and the stem cell functionality could be mechanistically related. By comparing newborns, we could demonstrate that telomerase re-introduction recovered the small body size of late generation telomerase-deficient mice. The same happened when we studied the body weight of age-matched adult mice of both genotypes suggesting a correlation between stem cell functionality and body-size.

11.1.5. Life span, cancer, and degenerative pathologies

Samper *et al.* (2001) demonstrated that mice with re-introduced telomerase exhibited nearly no adverse pathologies 21 days post-partum. In contrast, their late generation telomerase-deficient littermates had developed bone marrow aplasia, dramatically reduced spleen size, as well as different degrees of intestinal atrophy at the same age [65]. We examined the pathologies in aged mice and further demonstrated that the late generation telomerase-deficient mice with re-introduced telomerase exhibited a similar low incidence of degenerative pathologies when compared to normal heterozygous mice. All of the late generation telomerase-deficient mice developed degenerative pathologies, especially atrophy in the small intestine, very early in life. Importantly, the level of tumorigenesis was similar in normal heterozygous mice and late generation telomerase-deficient mice with re-introduced telomerase. An explanation for the small difference in tumorigenesis in these two genotypes could be due to chromosomal instability, breaks and fragments, in the reconstituted mice.

In agreement with the pathological finding, the survival rate of the telomerase-reconstituted mice is identical to the survival rate of wild-type and normal heterozygous controls. The life span of late telomerase-deficient mice is severely decreased, confirming already published results. Interestingly,

comparing late generation telomerase-deficient mice, littermates of the mice with re-introduced telomerase, to "normal" late generation telomerase-deficient mice we could not confirm a prolongation of the lifespan. We investigated this to find out if the re-introduction of a set of chromosomes with a normal telomere length inherited from the heterozygous parent into late generation telomerase-deficient mice with critically short telomeres would represent an advantage. Our findings did not confirm that and showed that survival of these mice is rather marked by the consequences of critically short telomeres.

As telomere-length homeostasis during the life span is important for health and longevity, we tested telomere length in different tissues of old mice heterozygous for telomerase and telomerase-reconstituted mice. We found that the mean telomere length and the percentages of short and long telomeres in mice reconstituted with telomerase activity are identical to that of normal heterozygous mice. Importantly, this result confirms that it is possible to obtain a normal balance in telomere length during life by re-introduction of telomerase.

Taken together, these data underscore the potential for application of therapies based on telomerase reactivation in premature aging diseases, like some cases of dyskeratosis congenital, aplastic anemia and idiopathic pulmonary fibrosis. Drawing from the conclusion of the results in mice, most probably, the defective tissue renewal capacity and increased cancer incidence of these patients can be combated successfully by this kind of treatment.

11.2. Deficient mismatch repair improves organismal fitness and survival of mice with dysfunctional telomeres

For a long time it was generally accepted that telomeres are structures that are protected against becoming substrates for DNA repair or recombination. However, it was recently discovered that components of the repair and recombination machineries are fundamental for telomere replication, protection and stability.

Rizki and Lundblad (2001) linked the mismatch repair (MMR) pathway to telomere-maintenance mechanisms [13]. Kucherlapati et al. (2001) proposed that defects in mismatch repair can, on one hand, provoke an accumulation of mutations due to defective DNA repair and, on the other hand, cause enhanced telomere maintenance by recombination at telomeres. Both of these mechanisms could contribute to cancer onset [186]. Additionally, MMR proteins are involved in DNA damage signaling and this in turn could have an effect on tumor development [16, 17, 175]. To clarify these observations, we characterized PMS2-deficient mice with dysfunctional telomeres. In these studies we also aimed to explain the role of MMR proteins in mediating the cellular response to critically short

telomeres. A role for MMR proteins in DNA damage signaling has been suggested in several studies [16, 17, 175].

11.2.1. Short telomeres as potent tumor suppressors

A previous report demonstrated that PMS2-deficieny resulted in an increased susceptibility to cancer [187]. Notably, increasing generations of Terc$^{-/-}$/PMS2$^{-/-}$ mice showed a gradually reduced tumor incidence when compared with single PMS2$^{-/-}$ mice. These results are in agreement with previous findings that suggest short telomeres may be potent tumor suppressors in a telomerase-deficient background [55, 188]. Importantly, telomerase deficiency rescues the survival defect of PMS2-deficient mice together with a decrease in tumorigenesis. To examine if telomerase inhibition may impede tumor growth, González-Suárez et al. (2000) studied tumor development in late-generation Terc$^{-/-}$ mice using multi-stage carcinogenesis [24]. Strikingly, telomerase-deficient mice with short telomeres were more resistant to skin tumorigenesis [55]. These findings agree with the results we obtained with the Terc/PMS2 mice, suggesting that tumors with PMS2 mutations may be successfully treated using telomerase inhibitors.

11.2.2. Degenerative pathologies in Terc/PMS2 mice

Han-Woong Lee et al. (1998) demonstrated that telomerase deficiency has a harmful effect on reproduction and haematopoiesis. Short telomeres, aberrant cytogenetic profiles, and affected cell renewal indicate a function for telomerase in cellular homeostasis [51]. Herrera et al. (1999) described several diseases associated with reduced telomere length, including splenic atrophy, abnormal blood cell counts and small intestinal atrophy [25]. Intriguingly, we found that PMS2 deficiency ameliorates severe degenerative pathologies provoked by short telomeres in all three generations of telomerase deficient mice. Additionally, the deficiency of this mismatch repair protein enhances the survival of telomerase-deficient mice. Which mechanism could explain these observations? There are several possible explanations for the observed higher fitness of these mice, including changes in telomere length, an improvement of telomere dysfunction, diminished microsatellite instability or alterations in the DNA damage response.

11.2.3. Which cellular mechanism is responsible for the increased fitness of the PMS2-deficient mice without telomerase?

The rescue of degenerative pathologies in mice deficient for PMS2 and telomerase is concomitant with a recuperation of proliferation defects in the small intestine of these mice. This result agrees with the

finding in yeast, that as a consequence of loss of the mismatch-repair function, cellular proliferation in the absence of telomerase is enhanced [13]. Recombination between telomeric sister chromatids is a mechanism by which cells could maintain long-term proliferation in the absence of telomerase [38, 40, 45, 189, 190]. Due to the anti-recombination activity of the MMR machinery, proliferation of telomerase-defective cells would be inhibited. Rizki *et al.* (2001) examined the growth of an *est2-Δ* strain, with a deletion for the catalytic subunit of telomerase, and compared it to an *est2-Δ msh2-Δ* double-mutant strain. Importantly, they observed a clear growth advantage of the double-mutant strain [13]. When they measured telomere length, they did not find a difference between strains with and without *MSH2*. The same results were obtained with strains containing mutations for MLH1 or PMS1 combined with a telomerase deficiency. In the case of MSH3 and MSH6, only double mutants displayed enhanced survival due to their partly redundant roles [13]. Severe proliferative defects and increased apoptosis result in the decreased survival of late generation telomerase-deficient mice, organs characterized by a high regeneration rates, like the gastrointestinal (GI) tract, are especially affected [25, 51]. However, in mice deficient for both PMS2 and telomerase, the cellular proliferation improved, in agreement with the results obtained by Rizki and Lundblad (2001) in yeast. Remarkably, the level of apoptosis did not differ in telomerase-deficient mice with or without PMS2 indicating that PMS2 deficiency does not have an effect on apoptosis.

11.2.4. Are proliferation defects rescued by maintaining telomere length or repairing telomere capping defects?

The improved fitness of the $Terc^{-/-}$/$PMS2^{-/-}$ mice could be explained by a maintenance or a lengthening of the telomeres. Nevertheless, telomere length was measured using 2 different methods, quantitative fluorescence *in situ* hybridization (Q-FISH) and southern-blot-based "terminal restriction fragment" (TRF) analysis. We did not detect an increase in telomere length, that would have explained the higher survival and amelioration in pathologies in the double knock-out mice. A known characteristic of cells employing alternative lengthening of telomeres (ALT), instead of telomerase, is a very heterogenous telomere length distribution. However, there was no evidence of telomere length heterogeneity in MEFs of double deficient mice. In order to completely exclude the activation of ALT, telomeric sister chromatid exchange (T-SCE) rates were examined. In yeast, *est2-Δ msh2-Δ* double-mutants that had re-acquired healthy growth exhibited both classes of characteristic telomeric and subtelomeric rearrangements observed previously in telomerase-defective survivor strains [13, 45]. Bechter et al. (2004) inhibited telomerase in a MMR-deficient cancer cell line and discovered that the telomeres were elongated in the absence of telomerase activity. In their case, they could correlate this telomere elongation with increased homologous recombination between the telomeres of sister chromatids. Additonally, they report that telomeric-sister chromatid exchange is characteristic of all established ALT cells they analyzed [14]. Previous studies have demonstrated an increased T-SCE in telomerase-

deficient keratinocytes and MEFs [42, 191]. We found T-SCE in telomerase deficient MEFs but no further increase related to an additional defect in PMS2. This result contradicts the previously proposed role for MMR genes in controlling telomeric recombination and telomere length and indicates that activation of ALT is not responsible for the PMS2 mediated rescue of proliferation defects in telomerase-deficient mice.

We next sought to test weather the increased proliferation was due to a decrease in DNA damage in the doubly-deficient mice. To measure this, we quantified the cells positive for γ-H2AX in the small intestine. γ-H2AX foci are an indication of DNA damage due to double-strand breaks and critically short and dysfunctional telomeres [73, 192]. As expected, the level of DNA damage increased with augmenting generations of telomerase deficiency, however we could not find a difference between mice singly deficient for telomerase and the double deficient mice. These results were confirmed by cytogenetic analysis of end-to-end fusions and signal-free ends, aberrations typically associated with telomerase deficiency. Again, the frequency of aberrations, and therefore DNA damage, was similar in telomerase deficient and in double deficient MEFs.

Microsatellite instability (MSI) is closely linked to defects in mismatch repair and is associated with increased cancer in mice [193]. Hence, we studied whether we could find an improvement in the frequency of MSI that could explain the lower cancer incidence in the double mutant mice. Again, we could not see a difference between double-deficient mice and mice deficient in telomerase alone. An unexpected result was the progressive increase in microsatellite instability with increasing generations of telomerase deficiency. Telomere shortening seems to lead to microsatellite instability, an intriguing result that will be the subject of future analysis.

11.2.5. Does PMS2 have a role in the cellular response to short and dysfuntional telomeres?

In the published literature there is evidence for an additional role of MMR in signaling DNA damage [16, 17, 175, 194, 195]. In one paper, it was found that MMR proteins are required for p53 phosphorylation in response to DNA methylation damage [175]. In MSH2-deficient cells the level of UVB-induced apoptosis and p53 phosphorylation at serine 15 are dramatically decreased [17]. hMLH1 is necessary for the accumulation of p73, a p53-related transcription factor, induced by cisplatin [194]. Other reports implicate MMR proteins directly in DNA damage signaling through the p53 pathway [16]. Additionally, the MMR proteins MLH1 and PMS2 are stimulated by p53 in response to DNA damage. Their first introns contain p53-response elements. Thus, it is possible that these genes function in cellular choice between cell-cycle arrest and apoptosis [195].

Telomere dysfunction results in DNA damage signaling and consequently in cell cycle arrest, senescence or apoptosis [51, 196, 197]. p21 is one of the downstream targets of p53. An upregulation of p21 provokes cell cycle arrest during replicative senescence in cell culture [198, 199]. Interestingly, the lifespan of primary human cells increases upon removal of *Trp53*, which encodes p53 [196]. However, genomic instability and tumor development negatively influence the life span of mice with dysfunctional telomeres in the absence of p53 [83, 200]. Choudhury et al. (2007) showed that *Cdkn1a* deletion ameliorates stem cell function and the lifespan of mice with dysfunctional telomeres without accelerating cancer formation [78]. Abrogation of Exonuclease-1 (EXO1), a 5´- 3´exonuclease involved in the MMR signaling pathway, was also able to delay aging pathologies in telomerase-deficient mice by rescuing proliferative defects associated with short telomeres [201]. These mice display an improved organ maintenance and longer lifespan compared to single telomerase-deficient mice [201]. Additionally, deletion of EXO1 reduces the accumulation of DNA damage and DNA damage signal induction in response to short telomeres [201]. We analyzed the p21 levels in small intestine and MEFs to ascertain if the enhancement of survival of telomerase deficient mice mediated by PMS2 deficiency could be linked to a role for PMS2 in signaling dysfunctional telomeres. The phenotype of mice deficient for both telomerase and PMS2 is strikingly similar to the phenotype of p21-deficient mice with short telomeres. Excitingly, we found that PMS2 deficiency results in an attenuated p21 induction in mice with short telomeres. This attenuated p21 induction is accompanied by a rescue of proliferation impairment but, importantly, it is not associated with changes in apoptosis level. Tumorigenesis is not enhanced and the $Terc^{-/-}/PMS2^{-/-}$ mice live longer. All of these observations coincide with the phenotype of the $Terc^{-/-}/p21^{-/-}$ mice. Based on these findings, it can be concluded that PMS2 and p21 may be in the same pathway signaling cell cycle arrest associated with telomere dysfunction. Additionally, these findings support the idea that cell cycle arrest may be dominant over apoptosis in causing age-related pathologies in telomerase-deficient mice. Most importantly, taking these results together with findings of other work it is possible to separate the pro-aging effect of short telomeres – PMS2- and p21-dependent - from the anticancer effect of short telomeres – p53-dependent but p21-independent.

12. CONCLUSIONS

12.1. Telomerase reverses epidermal hair follicle stem cell defects and loss of long-term survival associated with critically short telomeres

The work described in this thesis demonstrates that restoration of telomerase activity in late generation telomerase-deficient mice recuperates telomerase-associated defects and leads to normal stem cell behavior in epidermal hair follicles (Fig. 1). As a consequence of this recovery, the skin regains normal functionality, in contrast to the impaired skin functions of the late telomerase deficient mice, which manifest as premature aging of the hair and the skin. In summary, stem cell mobilization defects due to telomerase deficiency are corrected, leading to recuperation of hair growth and restoration of the hyperplasic response is normal.

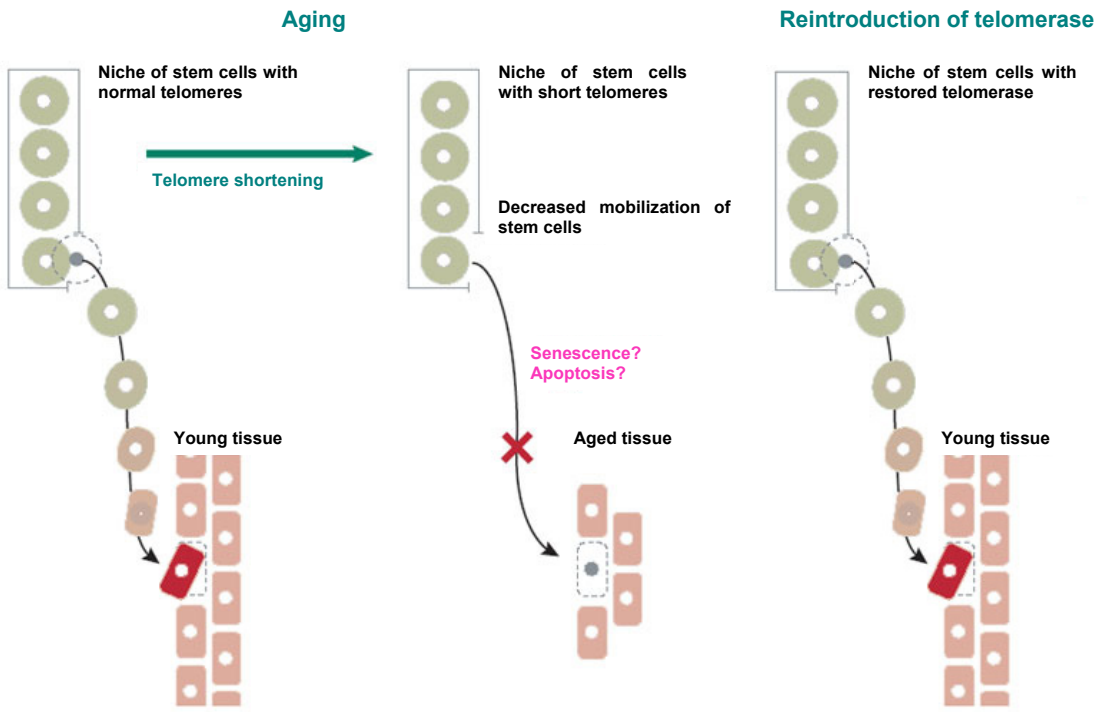

Figure 1: Left, normal **tissue regeneration by stem cells** with normal telomere length and therefore, regular stem cell mobilization. Middle, decreased stem cell mobilization caused by short telomeres related to aging. Right, normal stem cell mobilization out of the niche and tissue regeneration after re-introducing telomerase activity [29].

Telomerase is seen as a "stem cell factor" as it is noticeably expressed in several of the stem cell compartments. In the telomerase-deficient mouse model, stem cell function is defective in several tissues, including bone marrow, brain, and skin. Restoration of telomerase activity abolishes the stem

cell defects and is consistent with the hypothesis that stem cell function is dependent on telomere length [56].

When keratinocyte differentiation was assessed through the differentiation markers K14 and p63 we found no differences between late generation telomerase-deficient mouse skin and mouse skin with re-introduced telomerase. Telomerase re-introduction negates the appearance of chromosomal aberrations associated with short telomeres, like "signal-free" ends, rings, and end-to-end chromosome fusions. This finding strongly supports the notion that in late generation telomerase-deficient mice stem cell defects are associated with increased chromosomal damage. We did not find apoptotic cells in the skin of either of the two genotypes. Therefore we excluded the possibility that defective cells were removed by apoptosis and that normal cells took over and propagated. The level of proliferation in skin keratinocytes is similar both with and without re-introduced telomerase. It therefore seems that stem cell defects are apparent before severe proliferative defects can be detected.

An additional effect of telomerase restoration is a normal long-term organismal life span through the attenuation of premature aging, which is likely to be related to normal stem cell function. Fundamentally, telomerase was re-introduced without increasing the cancer incidence in mice. These findings are of critical importance for the development of therapies for premature aging diseases characterized by impaired telomerase activity and short telomeres. A hallmark of these diseases, including some cases of dyskeratosis congenita (DKC), idiopathic pulmonary fibrosis and aplastic anemia, is a defective tissue renewal capacity, especially in bone marrow and skin, as well as an increased cancer incidence [174]. The aim of this project was to obtain a "normal" state of telomere biology in the mice, avoiding critical telomere shortening on one hand and preventing aberrant telomere elongation on the other. We were able to demonstrate that by reintroducing telomerase normal telomere-length homeostasis during the life span of these mice could be achieved.

Importantly, we showed that the effects of telomere length and telomerase activity on different stem cell compartments are cell-autonomous. This finding is fundamental for the development of therapies based on telomerase reactivation. Furthermore, telomerase re-introduction corrects the defective *ex vivo* proliferative capacity of epidermal stem cells from late generation telomerase-deficient mice. Thus we can conclude that the effect of telomerase and telomere length are not related to factors in the stem cell niche, but are intrinsic to the stem cells.

12.2. Deficient mismatch repair improves organismal fitness and survival of mice with dysfunctional telomeres

Depletion of PMS2 in yeast strains lacking telomerase recuperates the survival and proliferation defects caused by the absence of telomerase. Based on these results, it was concluded that MMR genes are involved in telomeric sister chromatid recombination and by extension, in alternative telomere lengthening. Rizki and Lundblad (2001) suggested that the mismatch-repair machinery is responsible for the inhibition of recombination between non-identical telomeric sequences [13]. We therefore wished to examine if telomere length is maintained in the absence of telomerase upon the abolishment of the mismatch repair.

In our experiments in PMS2 deficienct mice with dysfuntional telomeres we obtained overall similar survival results to Rizki and Lundblad (2001) in yeast. The potent tumor suppressor function of short telomeres was already known [55, 188]. Strikingly, the level of tumor incidence caused by PMS2 deficiency is greatly reduced in mice with short telomeres, thereby positively influencing their lifespan. Hence, telomerase inhibitors could be promising as a therapeutic agent in the treatment of tumors with PMS2 mutations. Additionally, degenerative pathologies related to short telomeres were also improved in PMS2 null mice.

In PMS2 deficient mice with dysfunctional telomeres the prolonged lifespan coincided with an amelioration of proliferation in the GI tract. Until now, the results of the study in yeast paralleled with the findings in mice. However, we did not observe any change in telomere length nor find differences in sister telomeric recombination that would indicate that higher survival was due to telomere maintenance by ALT. PMS2 deficiency did not have an effect on the DNA damage load, either.

Another explanation for the observed phenotype would be a role for PMS2 in the cellular response to short and dysfunctional telomeres. Interestingly, we found that PMS2 deficiency led to an attenuated p21 response in telomerase deficient mice. Importantly, PMS2 deficiency in the context of short telomeres recuperated cellular proliferation defects in the absence of changes in the level of apoptosis.

The phenotype of the *Terc*$^{-/-}$*/PMS2*$^{-/-}$ mice resembles the phenotype of the *Terc*$^{-/-}$*/p21*$^{-/-}$ mice. These animals live longer and do not demonstrate increased tumorigenesis and their phenotype is independent of telomere length changes and telomeric dysfunction. Proliferation defects caused by telomere shortening are reduced in the absence of p21, and similar to our results with PMS2, the level of apoptosis is not affected. In contrast, p53 deficiency does not prolong the lifespan of telomerase-deficient mice and both proliferative and apoptotic defects are avoided. In these mice, tumors develop rapidly. p21 induces cell-cycle arrest and is transcriptionally activated by p53. Nevertheless, recent

findings indicate that p21 is not a critical effector of the tumor-suppressive capacities of p53. The observed similiarities with p21 suggest a similar role for PMS2. It is tempting to speculate that this mismatch repair protein may act in the same pathway as p21, mediating cell-cycle arrest and aging provoked by telomere shortening (Fig. 2). A separation of the age-promoting and tumor-suppressive activities of p53 would be an important step forward in the treatment of cancer and age-related diseases.

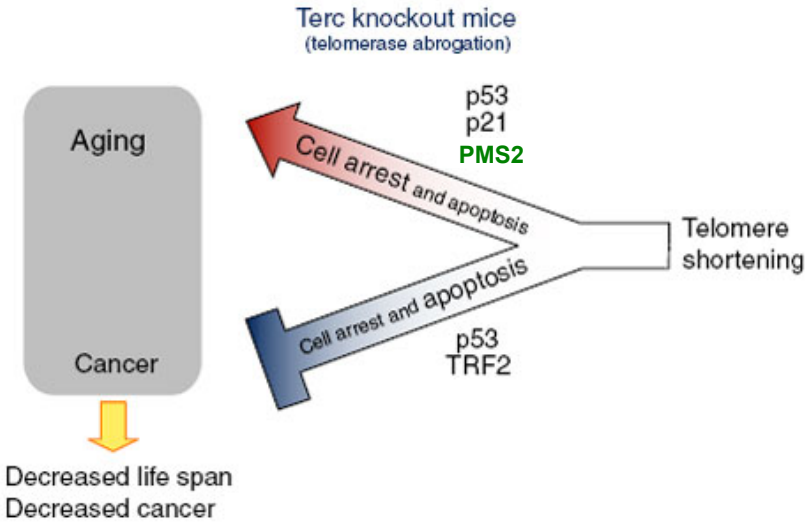

Figure 2: The telomerase knockout mouse as a model for telomere-induced aging (closer explanation see Fig. 8, chapter 3.3.1.). Interestingly, p21 and PMS2 deficiency in mice with short telomeres results in an improvement of proliferative defects but not of apoptosis [29].

Figure 3 describes a model for the role of the mismatch repair protein PMS2 in the mediation of the cellular response to short and dysfunctional telomeres. The G-strand overhang of the telomere forms the D-loop by strand invasion and pairing with the complementary sequence of the telomere, added by telomeric proteins. This structure is formed by telomeres of a normal length. However, short telomeres could not form the D-loop in the telomeric region, but may pair with subtelomeric region, thereby forming mismatches due to the reduced complimentarity of the sequences. This unusual mismatch cannot be corrected by mismatch repair itself. Consequently the p53/p21 pathway is induced and the cell cycle arrests.

This new model raises many interesting questions, but it needs to be confirmed experimentally. Mismatch repair proteins bound to the mismatched sequence could be identified using Chip analysis, but the structure of the mismatched D-loop remains to be determined. This new model will be the basis of future studies and will open new and exciting directions of research in the field of DNA repair and telomeres.

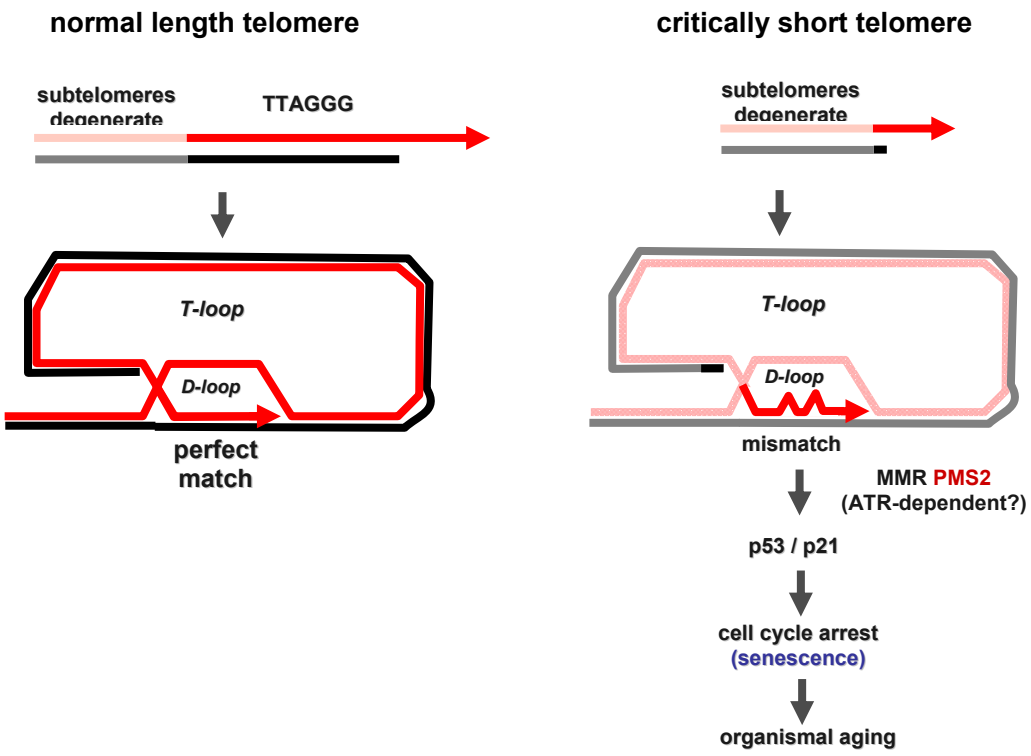

Figure 3: Model explaining the role of the mismatch repair protein PMS2 in telomere biology. The T-loop structure of normal length telomeres is shown on the left side. The G-strand overhang can form the D-loop because it perfectly matches with the complementary sequence on the telomere. On the right, a model for the situation of critically short telomeres. Mismatches are produced by defective pairing of two non-complementary sequences and cell cycle arrest is induced.

13. CONCLUSIONES

13.1. La telomerasa revierte los defectos de las células madre del folículo piloso epidérmico y la pérdida de la supervivencia a largo plazo asociada a los telómeros críticamente cortos

El trabajo descrito en esta tesis demuestra que la restauración de la actividad telomerasa en las generaciones tardías de ratones deficientes en la telomerasa recupera los defectos asociados a la telomerasa y conduce a un comportamiento normal de las células madre de los folículos pilosos de la epidermis (Fig. 1). Como consecuencia de esta recuperación, la piel recobra su funcionalidad normal, lo que contrasta con las funciones cutáneas que se encuentran impedidas en los ratones de generaciones tardías deficientes en telomerasa, los cuales manifiestan un envejecimiento prematuro del cabello y la piel. En resumen, se corrigen los defectos en la movilización de las células madre que habían sido ocasionados por la deficiencia en la telomerasa, lo que conduce a la recuperación del crecimiento capilar y a la restauración de una respuesta hiperplásica normal.

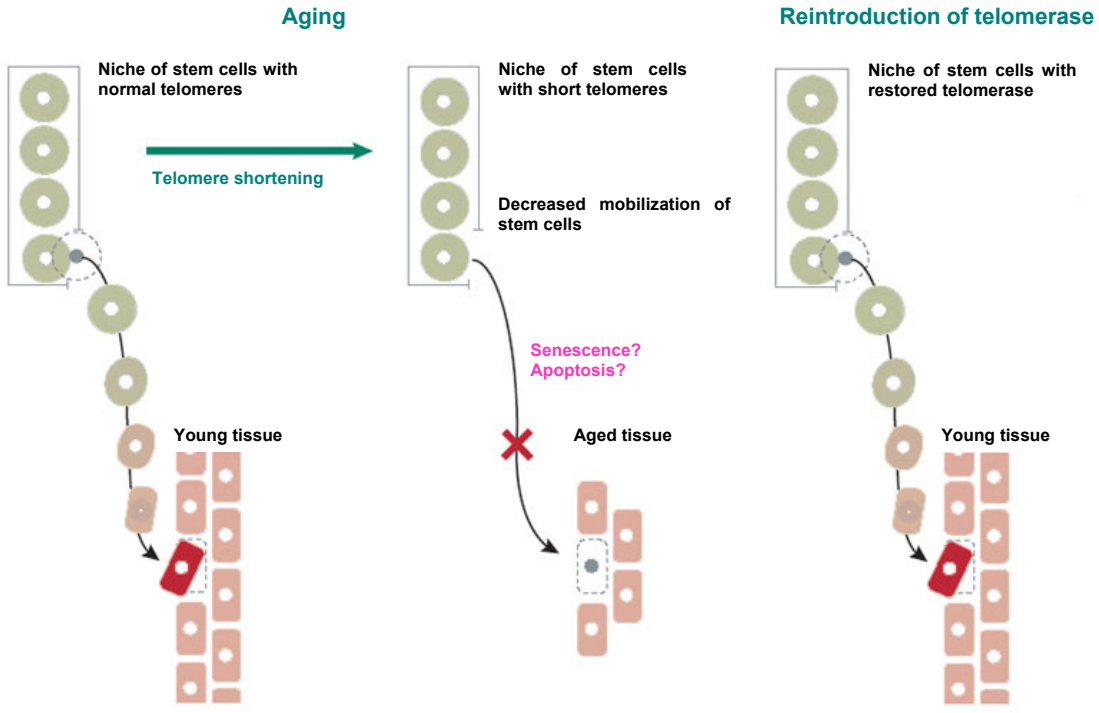

Figura 1. Izquierda, regeneración tisular normal ejercida por células made de longitud telomérica normal y, por tanto, una movilización de células madre regular. En medio, movilización de las células madre disminuida por la presencia de telómeros cortos asociados al envejecimiento. Derecha, las células madre abandonan sus nichos de forma normal tras la reintroducción de la actividad de la telomerasa [29].

La telomerasa es percibida como un "factor de células madre" ya que se expresa de forma notable en varios de los compartimentos de las células madre. En el modelo murino deficiente en telomerasa, la función de las células madre es defectuosa en varios tejidos, entre los que se encuentran la médula ósea, el cerebro y la piel. La restauración de la actividad telomerasa abole de los defectos de las células madre y es consistente con la hipótesis de que la funcionalidad de las células madre depende de la longitud telomérica [56].

No encontramos ninguna diferencia entre las pieles de los ratones de generaciones tardías deficientes en telomerasa y las de los ratones en los que se había reintroducido la telomerasa cuando evaluamos la diferenciación de queratinocitos mediante los marcadores de diferenciación K14 y p63. La reintroducción de la telomerasa impide la aparición de las aberraciones cromosómicas asociadas a los telómeros cortos, como los extremos "sin señal", los anillos y las fusiones de cromosomas entre extremos. Este hallazgo apoya con contundencia la noción de que en los ratones de generaciones tardías deficientes en telomerasa los defectos en las células madres están asociados a un aumento en el daño cromosómico. No detectamos células cutáneas apotóticas en ninguno de los dos genotipos. Excluimos, por lo tanto, la posibilidad de que las células defectuosas hayan sido eliminadas mediante apoptosis y reemplazadas por células normales que se hayan propagado. El nivel de proliferación en los queratinocitos cutáneos es similar, tanto en los que se ha reintroducido la telomerasa como en los que no. Parece entonces que los defectos en las células madre aparecen con anterioridad a que se puedan detectar los defectos proliferativos severos.

Un efecto adicional de la restauración telomérica es una expectativa de vida normal del organismo a largo plazo, gracias a la atenuación del envejecimiento prematuro, que con probabilidad estaría relacionado con una funcionalidad normal de las células madre. Es fundamental puntualizar que la telomerasa se reintrodujo sin aumentar la incidencia del cáncer en los ratones. Estos hallazgos tienen una importancia crítica para el desarrollo de terapias para las enfermedades de envejecimiento prematuro caracterizado por una actividad telomerasa impedida y unos telómeros cortos. Una característica básica de estas enfermedades, entre las que se encuentran la disqueratosis congénita (DKC), la fibrosis pulmonar idiopática y la anemia aplásica, es una capacidad de renovación tisular disminuida, especialmente en la médula ósea y en la piel, así como un aumento en la incidencia del cáncer [174]. El objetivo de este proyecto era la obtención de un estado "normal" de la biología de los telómeros en ratones, evitando, por un lado, el acortamiento crítico de telómeros y previniendo el alargamiento telomérico aberrante, por otro. Hemos sido capaces de demostrar que gracias a la reintroducción de la telomerasa se puede alcanzar una homeostasis normal en la longitud de los telómeros durante el período de vida de estos ratones.

Es importante resaltar que hemos mostrado que los efectos de la longitud telomérica y la actividad telomerasa sobre los diferentes compartimentos de células madre son autónomos de las células. Este hallazgo es fundamental para el desarrollo de terapias basadas en la reactivación de la telomerasa. Es más, la reintroducción de la telomerasa en las generaciones tardías de los ratones deficientes en telomerasa corrige los defectos en la capacidad proliferativa *ex vivo* de las células madres epidérmicas. Podemos concluir entonces que el efecto de la telomerasa y la longitud de los telómeros no están relacionados con los factores del nicho de las células madre, sino que son intrínsecos a éstas.

13.2. Una reparación deficiente de los errores de apareamiento mejora el estado de forma del organismo y la supervivencia de ratones con telómeros no funcionales

La disminución de PMS2 en cepas de levaduras que carecen de telomerasa recuperan los defectos en la supervivencia y la proliferación que causa la ausencia de la telomerasa. Basandose en los resultados anteriores, se concluyó que los genes de MMR están involucrados en la recombinación de las cromátidas hermanas y por extensión, en el alargamiento alternativo de los telómeros. Rizki y Lundblad (2002) sugirieron que la maquinaria de reparación de los apareamientos erróneos es la responsable de la inhibición de la recombinación entre secuencias teloméricas no idénticas [13]. Deseábamos por tanto examinar si la longitud telomérica se mantenía en ausencia de la telomerasa tras la eliminación de la vía de reparación del apareamiento de errores.

En nuestros experimentos con ratones deficientes en PMS2, que tienen telómeros no funcionales, obtuvimos unos resultados globales de supervivencia similares a los de Rizki y Lundblad (2001) en levaduras. Ya era conocida la potente funcionalidad supresora de tumores de los telómeros cortos [55, 188]. Llamativamente, en los ratones con telómeros cortos el nivel de la incidencia de tumores que causa la deficiencia en PMS2 está muy reducido, lo que influye positivamente en el periodo de vida. Por tanto, los inhibidores de la telomerasa podrían ser unos prometedores agentes en el tratamiento de tumores con mutaciones en PMS2. Adicionalmente, las patologías degenerativas relacionadas con los telómeros cortos también mejoraban en los ratones nulos para PMS2.

En los ratones deficientes en PMS2 con telómeros no funcionales la prolongación en la vida coincidía con una mejoría en la proliferación de las células del tracto gastrointestinal. Hasta ahora, los resultados de los estudios en levaduras eran paralelos a los hallazgos en ratones. Sin embargo, nosotros no hemos observado ningún cambio, ni en la longitud telomérica ni en la recombinación de las cromátidas hermanas, que indicarían que una mayor supervivencia era debida a un mantenimiento telomérico mediante ALT. La deficiencia en PMS2 tampoco tuvo un efecto sobre la tasa de daño en el ADN.

Otra explicación para el fenotipo observado sería la de una participación de PMS2 en la respuesta celular a los telómeros cortos y no funcionales. Es interesante puntualizar que encontramos que la deficiencia en PMS2 conducía a una respuesta atenuada de p21 en los ratones deficientes en telomerasa, y la deficiencia en PMS2, en el contexto de los telómeros cortos, recuperaba los defectos en la proliferación celular en ausencia de cambios en el nivel de apoptosis.

El fenotipo de los ratones *Terc*$^{-/-}$*/PMS2*$^{-/-}$ se asemeja al de los ratones *Terc-*$^{-/-}$*/p21*$^{-/-}$. Estos animales viven más y no muestran un aumento en la tumorigénesis; su fenotipo es independiente de los cambios en la longitud telomérica y de la falta de funcionalidad del telómero. Los defectos en la proliferación que causa el acortamiento telomérico se reducen en ausencia de p21 y, en forma similar a nuestros resultados con PMS2, el nivel de apoptosis no está afectado. Por el contrario, la deficiencia en p53 no prolonga la vida de los ratones deficientes en telomerasa y se evitan los defectos, tanto proliferativos como apoptóticos. En estos ratones, los tumores se desarrollan rápidamente; p21 induce la parada del ciclo celular y se activa transcripcionalmente por p53. Sin embargo, hay resultados recientes que indican que p21 no es un efector crítico de las capacidades de supresión tumoral de p53. Las similitudes que se han observado con p21 sugieren una función similar para PMS2. Es tentador especular que esta proteína de la reparación de los apareamientos erróneos pudiera ejercer en la misma ruta que p21, mediando la parada del ciclo celular y el envejecimiento provocado por el acortamiento telomérico (Fig. 2). La separación de las actividades de promoción del envejecimiento y de supresión tumoral de p53 sería un importante paso adelante hacia el tratamiento del cáncer y de las enfermedades relacionadas con el envejecimiento.

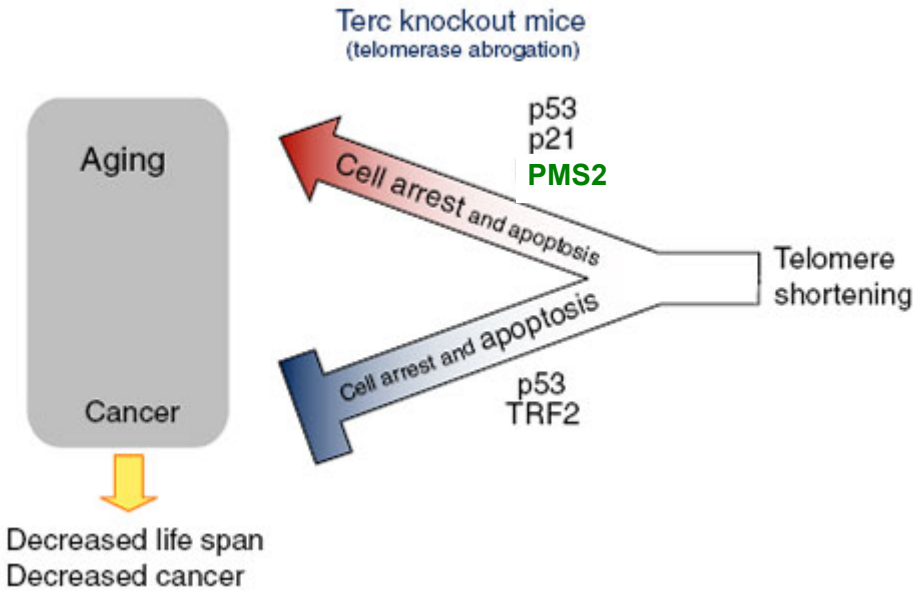

Figura 2: El ratón *knock-out* para la telomerasa como modelo del envejecimiento inducido por los telómeros (para una explicación más detallada consultar la Fig. 8, capítulo 3.3.1.). La deficiencia en p21 y PMS2 en ratones con telómeros cotos resulta en una mejoría en los defectos proliferativos, pero no en la apoptosis [29].

La Figura 3 describe un modelo para la funcionalidad de la proteína PMS2 en la reparación de los apareamientos erróneos en la mediación de la respuesta celular a los telómeros cortos y no funcionales. La hebra-G protuberante del telómero formaría un *D-loop* mediante la invasión de una cadena y el apareamiento con la secuencia complementaria del telómero, añadida por las proteínas teloméricas. Esta estructura se forma en telómeros de longitud normal. Los telómeros cortos, sin embargo, no podrían formar un *D-loop* en la región telomérica, pero podrían aparearse en la región subtelomérica, formando así apareamientos erróneos debido a la complementaridad reducida de las secuencias. Este desapareamiento inusual no se puede corregir mediante la propia maquinaria de reparación de los apareamientos erróneos, en consecuencia, se induce la ruta p53/p21 y se detiene el ciclo celular.

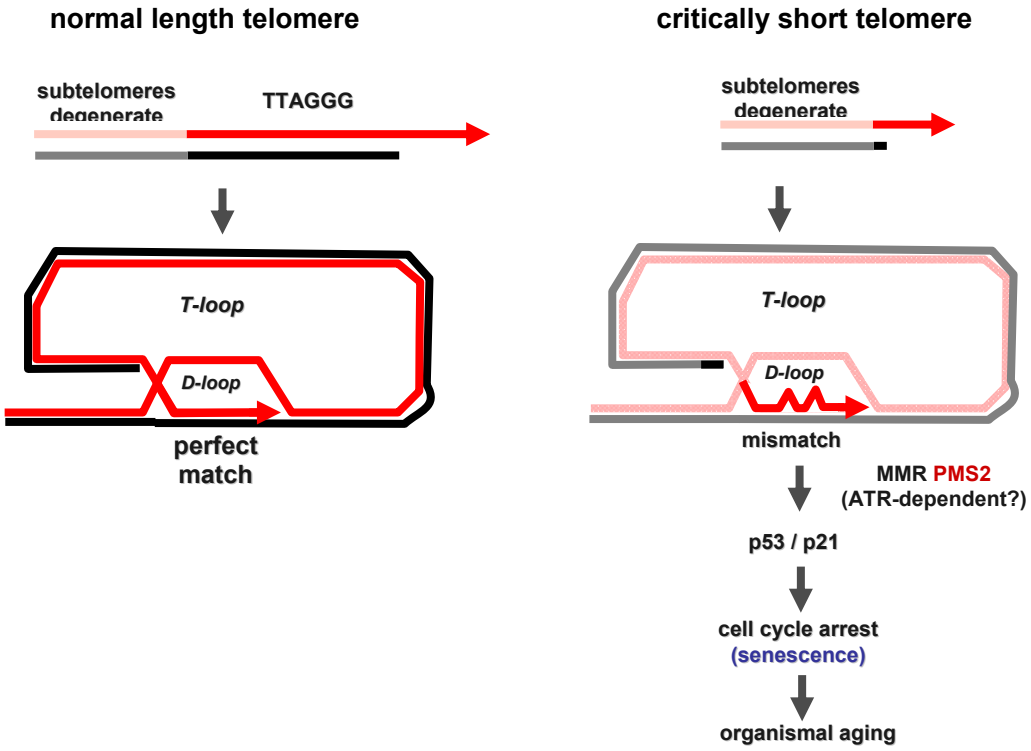

Figura 16: Modelo para explicar la función de la proteína PMS2 de reparación de apareamientos erróneos en la biología del telómero. Se muestra a la izquierda la estructura de *T-loop* de los telómeros de longitud normal. La hebra-G protuberante puede formar el *D-loop* porque complementa perfectamente la secuencia del telómero. A la derecha, un modelo para la situación de los telómeros críticamente cortos. Los apareamientos erróneos se producen por un apareamiento defectuoso de dos secuencias no complementarias y se induce la parada del ciclo celular.

De este nuevo modelo surgen preguntas interesantes, pero primero debe ser confirmado experimentalmente. No pudimos identificar mediante un análisis con ChIP las proteínas de la reparación de los apareamientos erróneos unidas a la secuencia donde se ha producido el error, aunque queda por determinar la estructura del *D-loop* del apareamiento erróneo. Este nuevo modelo constituye la base de estudios futuros y abrirá nuevas y excitantes vías de investigación en el campo de la reparación del ADN y los telómeros.

14. REFERENCES

1. de Lange, T., *Shelterin: the protein complex that shapes and safeguards human telomeres.* Genes Dev, 2005. **19**(18): p. 2100-10.
2. Blackburn, E.H., *Switching and signaling at the telomere.* Cell, 2001. **106**(6): p. 661-73.
3. Chan, S.W. and E.H. Blackburn, *New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin.* Oncogene, 2002. **21**(4): p. 553-63.
4. Mitchell, J.R., E. Wood, and K. Collins, *A telomerase component is defective in the human disease dyskeratosis congenita.* Nature, 1999. **402**(6761): p. 551-5.
5. Vulliamy, T., et al., *The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita.* Nature, 2001. **413**(6854): p. 432-5.
6. Vulliamy, T., et al., *Disease anticipation is associated with progressive telomere shortening in families with dyskeratosis congenita due to mutations in TERC.* Nat Genet, 2004. **36**(5): p. 447-9.
7. Marrone, A., et al., *Heterozygous telomerase RNA mutations found in dyskeratosis congenita and aplastic anemia reduce telomerase activity via haploinsufficiency.* Blood, 2004. **104**(13): p. 3936-42.
8. Yamaguchi, H., et al., *Mutations in TERT, the gene for telomerase reverse transcriptase, in aplastic anemia.* N Engl J Med, 2005. **352**(14): p. 1413-24.
9. Armanios, M.Y., et al., *Telomerase mutations in families with idiopathic pulmonary fibrosis.* N Engl J Med, 2007. **356**(13): p. 1317-26.
10. Tsakiri, K.D., et al., *Adult-onset pulmonary fibrosis caused by mutations in telomerase.* Proc Natl Acad Sci U S A, 2007. **104**(18): p. 7552-7.
11. Su, T.T., *Cellular responses to DNA damage: one signal, multiple choices.* Annu Rev Genet, 2006. **40**: p. 187-208.
12. Saydam, N., et al., *Physical and functional interactions between Werner syndrome helicase and mismatch-repair initiation factors.* Nucleic Acids Res, 2007. **35**(17): p. 5706-16.
13. Rizki, A. and V. Lundblad, *Defects in mismatch repair promote telomerase-independent proliferation.* Nature, 2001. **411**(6838): p. 713-6.
14. Bechter, O.E., et al., *Telomeric recombination in mismatch repair deficient human colon cancer cells after telomerase inhibition.* Cancer Res, 2004. **64**(10): p. 3444-51.
15. Duckett, D.R., et al., *Human MutSalpha recognizes damaged DNA base pairs containing O6-methylguanine, O4-methylthymine, or the cisplatin-d(GpG) adduct.* Proc Natl Acad Sci U S A, 1996. **93**(13): p. 6443-7.
16. Luo, Y., F.T. Lin, and W.C. Lin, *ATM-mediated stabilization of hMutL DNA mismatch repair proteins augments p53 activation during DNA damage.* Mol Cell Biol, 2004. **24**(14): p. 6430-44.
17. Peters, A.C., et al., *Mammalian DNA mismatch repair protects cells from UVB-induced DNA damage by facilitating apoptosis and p53 activation.* DNA Repair (Amst), 2003. **2**(4): p. 427-35.
18. Blasco, M.A., *Telomeres and human disease: ageing, cancer and beyond.* Nat Rev Genet, 2005. **6**(8): p. 611-22.
19. Blasco, M.A., *Mice with bad ends: mouse models for the study of telomeres and telomerase in cancer and aging.* Embo J, 2005. **24**(6): p. 1095-103.
20. de Lange, T., *Protection of mammalian telomeres.* Oncogene, 2002. **21**(4): p. 532-40.
21. Griffith, J.D., et al., *Mammalian telomeres end in a large duplex loop.* Cell, 1999. **97**(4): p. 503-14.
22. Nikitina, T. and C.L. Woodcock, *Closed chromatin loops at the ends of chromosomes.* J Cell Biol, 2004. **166**(2): p. 161-5.
23. de Lange, T., *T-loops and the origin of telomeres.* Nat Rev Mol Cell Biol, 2004. **5**(4): p. 323-9.
24. Blasco, M.A., et al., *Telomere shortening and tumor formation by mouse cells lacking telomerase RNA.* Cell, 1997. **91**(1): p. 25-34.

25. Herrera, E., et al., *Disease states associated with telomerase deficiency appear earlier in mice with short telomeres.* Embo J, 1999. **18**(11): p. 2950-60.
26. Lingner, J., J.P. Cooper, and T.R. Cech, *Telomerase and DNA end replication: no longer a lagging strand problem?* Science, 1995. **269**(5230): p. 1533-4.
27. Watson, J.D., *Origin of concatemeric T7 DNA.* Nat New Biol, 1972. **239**(94): p. 197-201.
28. Olovnikov, A.M., *A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon.* J Theor Biol, 1973. **41**(1): p. 181-90.
29. Blasco, M.A., *Telomere length, stem cells and aging.* Nat Chem Biol, 2007. **3**(10): p. 640-9.
30. Cohen, S.B., et al., *Protein composition of catalytically active human telomerase from immortal cells.* Science, 2007. **315**(5820): p. 1850-3.
31. Collins, K. and J.R. Mitchell, *Telomerase in the human organism.* Oncogene, 2002. **21**(4): p. 564-79.
32. Harley, C.B., A.B. Futcher, and C.W. Greider, *Telomeres shorten during ageing of human fibroblasts.* Nature, 1990. **345**(6274): p. 458-60.
33. Niida, H., et al., *Telomere maintenance in telomerase-deficient mouse embryonic stem cells: characterization of an amplified telomeric DNA.* Mol Cell Biol, 2000. **20**(11): p. 4115-27.
34. Flores, I., M.L. Cayuela, and M.A. Blasco, *Effects of telomerase and telomere length on epidermal stem cell behavior.* Science, 2005. **309**(5738): p. 1253-6.
35. Sarin, K.Y., et al., *Conditional telomerase induction causes proliferation of hair follicle stem cells.* Nature, 2005. **436**(7053): p. 1048-52.
36. Blasco, M.A., *The epigenetic regulation of mammalian telomeres.* Nat Rev Genet, 2007. **8**(4): p. 299-309.
37. Le, S., et al., *RAD50 and RAD51 define two pathways that collaborate to maintain telomeres in the absence of telomerase.* Genetics, 1999. **152**(1): p. 143-52.
38. Teng, S.C. and V.A. Zakian, *Telomere-telomere recombination is an efficient bypass pathway for telomere maintenance in Saccharomyces cerevisiae.* Mol Cell Biol, 1999. **19**(12): p. 8083-93.
39. Muntoni, A. and R.R. Reddel, *The first molecular details of ALT in human tumor cells.* Hum Mol Genet, 2005. **14 Spec No. 2**: p. R191-6.
40. Dunham, M.A., et al., *Telomere maintenance by recombination in human cells.* Nat Genet, 2000. **26**(4): p. 447-50.
41. Yeager, T.R., et al., *Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body.* Cancer Res, 1999. **59**(17): p. 4175-9.
42. Blanco, R., et al., *Telomerase abrogation dramatically accelerates TRF2-induced epithelial carcinogenesis.* Genes Dev, 2007. **21**(2): p. 206-20.
43. Wu, L., et al., *Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres.* Cell, 2006. **126**(1): p. 49-62.
44. Lundblad, V., *Telomere maintenance without telomerase.* Oncogene, 2002. **21**(4): p. 522-31.
45. Lundblad, V. and E.H. Blackburn, *An alternative pathway for yeast telomere maintenance rescues est1- senescence.* Cell, 1993. **73**(2): p. 347-60.
46. Laud, P.R., et al., *Elevated telomere-telomere recombination in WRN-deficient, telomere dysfunctional cells promotes escape from senescence and engagement of the ALT pathway.* Genes Dev, 2005. **19**(21): p. 2560-70.
47. Hande, M.P., et al., *Telomere length dynamics and chromosomal instability in cells derived from telomerase null mice.* J Cell Biol, 1999. **144**(4): p. 589-601.
48. Chang, S., et al., *Telomere-based crisis: functional differences between telomerase activation and ALT in tumor progression.* Genes Dev, 2003. **17**(1): p. 88-100.
49. Herrera, E., A.C. Martinez, and M.A. Blasco, *Impaired germinal center reaction in mice with short telomeres.* Embo J, 2000. **19**(3): p. 472-81.
50. Blasco, M.A., et al., *Functional characterization and developmental regulation of mouse telomerase RNA.* Science, 1995. **269**(5228): p. 1267-70.
51. Lee, H.W., et al., *Essential role of mouse telomerase in highly proliferative organs.* Nature, 1998. **392**(6676): p. 569-74.
52. Rudolph, K.L., et al., *Longevity, stress response, and cancer in aging telomerase-deficient mice.* Cell, 1999. **96**(5): p. 701-12.

53. Herrera, E., E. Samper, and M.A. Blasco, *Telomere shortening in mTR-/- embryos is associated with failure to close the neural tube.* Embo J, 1999. **18**(5): p. 1172-81.
54. Samper, E., et al., *Long-term repopulating ability of telomerase-deficient murine hematopoietic stem cells.* Blood, 2002. **99**(8): p. 2767-75.
55. Gonzalez-Suarez, E., et al., *Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis.* Nat Genet, 2000. **26**(1): p. 114-7.
56. Flores, I., R. Benetti, and M.A. Blasco, *Telomerase regulation and stem cell behaviour.* Curr Opin Cell Biol, 2006. **18**(3): p. 254-60.
57. Harrington, L., *Does the reservoir for self-renewal stem from the ends?* Oncogene, 2004. **23**(43): p. 7283-9.
58. Moore, K.A. and I.R. Lemischka, *Stem cells and their niches.* Science, 2006. **311**(5769): p. 1880-5.
59. Clarke, M.F. and M. Fuller, *Stem cells and cancer: two faces of eve.* Cell, 2006. **124**(6): p. 1111-5.
60. Serrano, M. and M.A. Blasco, *Cancer and ageing: convergent and divergent mechanisms.* Nat Rev Mol Cell Biol, 2007. **8**(9): p. 715-22.
61. Vulliamy, T., et al., *Association between aplastic anaemia and mutations in telomerase RNA.* Lancet, 2002. **359**(9324): p. 2168-70.
62. Dokal, I., *Dyskeratosis congenita in all its forms.* Br J Haematol, 2000. **110**(4): p. 768-79.
63. Armanios, M. and C.W. Greider, *Telomerase and cancer stem cells.* Cold Spring Harb Symp Quant Biol, 2005. **70**: p. 205-8.
64. Hiyama, E. and K. Hiyama, *Telomere and telomerase in stem cells.* Br J Cancer, 2007. **96**(7): p. 1020-4.
65. Samper, E., J.M. Flores, and M.A. Blasco, *Restoration of telomerase activity rescues chromosomal instability and premature aging in Terc-/- mice with short telomeres.* EMBO Rep, 2001. **2**(9): p. 800-7.
66. Alonso, L. and E. Fuchs, *Stem cells of the skin epithelium.* Proc Natl Acad Sci U S A, 2003. **100 Suppl 1**: p. 11830-5.
67. Gandarillas, A., *Epidermal differentiation, apoptosis, and senescence: common pathways?* Exp Gerontol, 2000. **35**(1): p. 53-62.
68. Potten, C.S., *Epidermal transit times.* Br J Dermatol, 1975. **93**(6): p. 649-58.
69. Mackenzie, I.C., *Retroviral transduction of murine epidermal stem cells demonstrates clonal units of epidermal structure.* J Invest Dermatol, 1997. **109**(3): p. 377-83.
70. Cotsarelis, G., T.T. Sun, and R.M. Lavker, *Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis.* Cell, 1990. **61**(7): p. 1329-37.
71. Alonso, L. and E. Fuchs, *The hair cycle.* J Cell Sci, 2006. **119**(Pt 3): p. 391-3.
72. Bodnar, A.G., et al., *Extension of life-span by introduction of telomerase into normal human cells.* Science, 1998. **279**(5349): p. 349-52.
73. d'Adda di Fagagna, F., et al., *A DNA damage checkpoint response in telomere-initiated senescence.* Nature, 2003. **426**(6963): p. 194-8.
74. Denchi, E.L. and T. de Lange, *Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1.* Nature, 2007. **448**(7157): p. 1068-71.
75. Shay, J.W. and W.E. Wright, *Telomerase therapeutics for cancer: challenges and new directions.* Nat Rev Drug Discov, 2006. **5**(7): p. 577-84.
76. Greenberg, R.A., et al., *Short dysfunctional telomeres impair tumorigenesis in the INK4a(delta2/3) cancer-prone mouse.* Cell, 1999. **97**(4): p. 515-25.
77. Rudolph, K.L., et al., *Telomere dysfunction and evolution of intestinal carcinoma in mice and humans.* Nat Genet, 2001. **28**(2): p. 155-9.
78. Choudhury, A.R., et al., *Cdkn1a deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation.* Nat Genet, 2007. **39**(1): p. 99-105.
79. Siegl-Cachedenier, I., et al., *Deficient mismatch repair improves organismal fitness and survival of mice with dysfunctional telomeres.* Genes Dev, 2007. **21**(17): p. 2234-47.

80. Espejel, S., et al., *Impact of telomerase ablation on organismal viability, aging, and tumorigenesis in mice lacking the DNA repair proteins PARP-1, Ku86, or DNA-PKcs.* J Cell Biol, 2004. **167**(4): p. 627-38.
81. Khoo, C.M., et al., *Ink4a/Arf tumor suppressor does not modulate the degenerative conditions or tumor spectrum of the telomerase-deficient mouse.* Proc Natl Acad Sci U S A, 2007. **104**(10): p. 3931-6.
82. Wong, K.K., et al., *Telomere dysfunction and Atm deficiency compromises organ homeostasis and accelerates ageing.* Nature, 2003. **421**(6923): p. 643-8.
83. Chin, L., et al., *p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis.* Cell, 1999. **97**(4): p. 527-38.
84. Maser, R.S., et al., *Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers.* Nature, 2007. **447**(7147): p. 966-71.
85. Bell, J.F. and N.E. Sharpless, *Telomeres, p21 and the cancer-aging hypothesis.* Nat Genet, 2007. **39**(1): p. 11-2.
86. Christophorou, M.A., et al., *The pathological response to DNA damage does not contribute to p53-mediated tumour suppression.* Nature, 2006. **443**(7108): p. 214-7.
87. Liu, D., et al., *Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins.* J Biol Chem, 2004. **279**(49): p. 51338-42.
88. Kim, S.H., P. Kaminker, and J. Campisi, *TIN2, a new regulator of telomere length in human cells.* Nat Genet, 1999. **23**(4): p. 405-12.
89. Smith, S. and T. de Lange, *Tankyrase promotes telomere elongation in human cells.* Curr Biol, 2000. **10**(20): p. 1299-302.
90. Smith, S., et al., *Tankyrase, a poly(ADP-ribose) polymerase at human telomeres.* Science, 1998. **282**(5393): p. 1484-7.
91. Cook, B.D., et al., *Role for the related poly(ADP-Ribose) polymerases tankyrase 1 and 2 at human telomeres.* Mol Cell Biol, 2002. **22**(1): p. 332-42.
92. Guglielmi, B. and M. Werner, *The yeast homolog of human PinX1 is involved in rRNA and small nucleolar RNA maturation, not in telomere elongation inhibition.* J Biol Chem, 2002. **277**(38): p. 35712-9.
93. Liu, D., et al., *PTOP interacts with POT1 and regulates its localization to telomeres.* Nat Cell Biol, 2004. **6**(7): p. 673-80.
94. Zhu, X.D., et al., *Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres.* Nat Genet, 2000. **25**(3): p. 347-52.
95. Bailey, S.M., et al., *DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 14899-904.
96. Ame, J.C., et al., *PARP-2, A novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase.* J Biol Chem, 1999. **274**(25): p. 17860-8.
97. Kaminker, P.G., et al., *TANK2, a new TRF1-associated poly(ADP-ribose) polymerase, causes rapid induction of cell death upon overexpression.* J Biol Chem, 2001. **276**(38): p. 35891-9.
98. Karlseder, J., et al., *Targeted deletion reveals an essential function for the telomere length regulator Trf1.* Mol Cell Biol, 2003. **23**(18): p. 6533-41.
99. Dynek, J.N. and S. Smith, *Resolution of sister telomere association is required for progression through mitosis.* Science, 2004. **304**(5667): p. 97-100.
100. Ye, J.Z. and T. de Lange, *TIN2 is a tankyrase 1 PARP modulator in the TRF1 telomere length control complex.* Nat Genet, 2004. **36**(6): p. 618-23.
101. Chiang, Y.J., et al., *Telomere-associated protein TIN2 is essential for early embryonic development through a telomerase-independent pathway.* Mol Cell Biol, 2004. **24**(15): p. 6631-4.
102. Kaminker, P., et al., *Higher-order nuclear organization in growth arrest of human mammary epithelial cells: a novel role for telomere-associated protein TIN2.* J Cell Sci, 2005. **118**(Pt 6): p. 1321-30.
103. Kim, S.H., et al., *TIN2 mediates functions of TRF2 at human telomeres.* J Biol Chem, 2004. **279**(42): p. 43799-804.
104. Ye, J.Z., et al., *TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres.* J Biol Chem, 2004. **279**(45): p. 47264-71.

105. Loayza, D. and T. De Lange, *POT1 as a terminal transducer of TRF1 telomere length control.* Nature, 2003. **423**(6943): p. 1013-8.
106. Smogorzewska, A. and T. de Lange, *Regulation of telomerase by telomeric proteins.* Annu Rev Biochem, 2004. **73**: p. 177-208.
107. Ye, J.Z., et al., *POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex.* Genes Dev, 2004. **18**(14): p. 1649-54.
108. Zhou, X.Z. and K.P. Lu, *The Pin2/TRF1-interacting protein PinX1 is a potent telomerase inhibitor.* Cell, 2001. **107**(3): p. 347-59.
109. Oh, B.K., et al., *Up-regulation of telomere-binding proteins, TRF1, TRF2, and TIN2 is related to telomere shortening during human multistep hepatocarcinogenesis.* Am J Pathol, 2005. **166**(1): p. 73-80.
110. Gelmini, S., et al., *Tankyrase, a positive regulator of telomere elongation, is over expressed in human breast cancer.* Cancer Lett, 2004. **216**(1): p. 81-7.
111. Kondo, T., et al., *Expression of POT1 is associated with tumor stage and telomere length in gastric carcinoma.* Cancer Res, 2004. **64**(2): p. 523-9.
112. Miyachi, K., et al., *Correlation between telomerase activity and telomeric-repeat binding factors in gastric cancer.* J Exp Clin Cancer Res, 2002. **21**(2): p. 269-75.
113. Matsutani, N., et al., *Expression of telomeric repeat binding factor 1 and 2 and TRF1-interacting nuclear protein 2 in human gastric carcinomas.* Int J Oncol, 2001. **19**(3): p. 507-12.
114. van Steensel, B., A. Smogorzewska, and T. de Lange, *TRF2 protects human telomeres from end-to-end fusions.* Cell, 1998. **92**(3): p. 401-13.
115. Munoz, P., et al., *XPF nuclease-dependent telomere loss and increased DNA damage in mice overexpressing TRF2 result in premature aging and cancer.* Nat Genet, 2005. **37**(10): p. 1063-71.
116. Zhu, X.D., et al., *ERCC1/XPF removes the 3′ overhang from uncapped telomeres and represses formation of telomeric DNA-containing double minute chromosomes.* Mol Cell, 2003. **12**(6): p. 1489-98.
117. Espejel, S., et al., *Mammalian Ku86 mediates chromosomal fusions and apoptosis caused by critically short telomeres.* Embo J, 2002. **21**(9): p. 2207-19.
118. Espejel, S., et al., *Functional interaction between DNA-PKcs and telomerase in telomere length maintenance.* Embo J, 2002. **21**(22): p. 6275-87.
119. Goytisolo, F.A., et al., *Short telomeres result in organismal hypersensitivity to ionizing radiation in mammals.* J Exp Med, 2000. **192**(11): p. 1625-36.
120. Stansel, R.M., T. de Lange, and J.D. Griffith, *T-loop assembly in vitro involves binding of TRF2 near the 3′ telomeric overhang.* Embo J, 2001. **20**(19): p. 5532-40.
121. Li, B., S. Oestreich, and T. de Lange, *Identification of human Rap1: implications for telomere evolution.* Cell, 2000. **101**(5): p. 471-83.
122. Li, B. and T. de Lange, *Rap1 affects the length and heterogeneity of human telomeres.* Mol Biol Cell, 2003. **14**(12): p. 5060-8.
123. Dantzer, F., et al., *Functional interaction between poly(ADP-Ribose) polymerase 2 (PARP-2) and TRF2: PARP activity negatively regulates TRF2.* Mol Cell Biol, 2004. **24**(4): p. 1595-607.
124. Song, K., et al., *Interaction of human Ku70 with TRF2.* FEBS Lett, 2000. **481**(1): p. 81-5.
125. Opresko, P.L., et al., *The Werner syndrome helicase and exonuclease cooperate to resolve telomeric D loops in a manner regulated by TRF1 and TRF2.* Mol Cell, 2004. **14**(6): p. 763-74.
126. Karlseder, J., et al., *The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response.* PLoS Biol, 2004. **2**(8): p. E240.
127. Takai, H., A. Smogorzewska, and T. de Lange, *DNA damage foci at dysfunctional telomeres.* Curr Biol, 2003. **13**(17): p. 1549-56.
128. Lustig, A.J., *Clues to catastrophic telomere loss in mammals from yeast telomere rapid deletion.* Nat Rev Genet, 2003. **4**(11): p. 916-23.
129. Sancar, A., et al., *Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints.* Annu Rev Biochem, 2004. **73**: p. 39-85.
130. Zhou, B.B. and S.J. Elledge, *The DNA damage response: putting checkpoints in perspective.* Nature, 2000. **408**(6811): p. 433-9.

131. Ataian, Y. and J.E. Krebs, *Five repair pathways in one context: chromatin modification during DNA repair.* Biochem Cell Biol, 2006. **84**(4): p. 490-504.
132. Nyberg, K.A., et al., *Toward maintaining the genome: DNA damage and replication checkpoints.* Annu Rev Genet, 2002. **36**: p. 617-56.
133. Melo, J. and D. Toczyski, *A unified view of the DNA-damage checkpoint.* Curr Opin Cell Biol, 2002. **14**(2): p. 237-45.
134. Banin, S., et al., *Enhanced phosphorylation of p53 by ATM in response to DNA damage.* Science, 1998. **281**(5383): p. 1674-7.
135. Canman, C.E., et al., *Activation of the ATM kinase by ionizing radiation and phosphorylation of p53.* Science, 1998. **281**(5383): p. 1677-9.
136. Lim, D.S., et al., *ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway.* Nature, 2000. **404**(6778): p. 613-7.
137. Cortez, D., et al., *Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks.* Science, 1999. **286**(5442): p. 1162-6.
138. Bakkenist, C.J. and M.B. Kastan, *DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation.* Nature, 2003. **421**(6922): p. 499-506.
139. Abraham, R.T., *Cell cycle checkpoint signaling through the ATM and ATR kinases.* Genes Dev, 2001. **15**(17): p. 2177-96.
140. Zou, L. and S.J. Elledge, *Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes.* Science, 2003. **300**(5625): p. 1542-8.
141. Yuzhakov, A., et al., *Multiple competition reactions for RPA order the assembly of the DNA polymerase delta holoenzyme.* Embo J, 1999. **18**(21): p. 6189-99.
142. Green, C.M., et al., *A novel Rad24 checkpoint protein complex closely related to replication factor C.* Curr Biol, 2000. **10**(1): p. 39-42.
143. O'Donnell, M., D. Jeruzalmi, and J. Kuriyan, *Clamp loader structure predicts the architecture of DNA polymerase III holoenzyme and RFC.* Curr Biol, 2001. **11**(22): p. R935-46.
144. Schultz, L.B., et al., *p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks.* J Cell Biol, 2000. **151**(7): p. 1381-90.
145. Wang, B., et al., *53BP1, a mediator of the DNA damage checkpoint.* Science, 2002. **298**(5597): p. 1435-8.
146. Yamane, K., X. Wu, and J. Chen, *A DNA damage-regulated BRCT-containing protein, TopBP1, is required for cell survival.* Mol Cell Biol, 2002. **22**(2): p. 555-66.
147. Goldberg, M., et al., *MDC1 is required for the intra-S-phase DNA damage checkpoint.* Nature, 2003. **421**(6926): p. 952-6.
148. Lou, Z., et al., *MDC1 is coupled to activated CHK2 in mammalian DNA damage response pathways.* Nature, 2003. **421**(6926): p. 957-61.
149. Stewart, G.S., et al., *MDC1 is a mediator of the mammalian DNA damage checkpoint.* Nature, 2003. **421**(6926): p. 961-6.
150. Rhind, N. and P. Russell, *Chk1 and Cds1: linchpins of the DNA damage and replication checkpoint pathways.* J Cell Sci, 2000. **113 (Pt 22)**: p. 3889-96.
151. McGowan, C.M., R.E. Posner, and R.M. Christley, *Incidence of exertional rhabdomyolysis in polo horses in the USA and the United Kingdom in the 1999/2000 season.* Vet Rec, 2002. **150**(17): p. 535-7.
152. Hirao, A., et al., *DNA damage-induced activation of p53 by the checkpoint kinase Chk2.* Science, 2000. **287**(5459): p. 1824-7.
153. Matsuoka, S., et al., *Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro.* Proc Natl Acad Sci U S A, 2000. **97**(19): p. 10389-94.
154. Zhao, H. and H. Piwnica-Worms, *ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1.* Mol Cell Biol, 2001. **21**(13): p. 4129-39.
155. Kunkel, T.A. and D.A. Erie, *DNA mismatch repair.* Annu Rev Biochem, 2005. **74**: p. 681-710.
156. Iyer, R.R., et al., *DNA mismatch repair: functions and mechanisms.* Chem Rev, 2006. **106**(2): p. 302-23.
157. Jiricny, J., *The multifaceted mismatch-repair system.* Nat Rev Mol Cell Biol, 2006. **7**(5): p. 335-46.
158. Peltomaki, P., *Lynch syndrome genes.* Fam Cancer, 2005. **4**(3): p. 227-32.

159. Peltomaki, P., *Role of DNA mismatch repair defects in the pathogenesis of human cancer.* J Clin Oncol, 2003. **21**(6): p. 1174-9.
160. Acharya, S., et al., *hMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13629-34.
161. Drummond, J.T., et al., *Isolation of an hMSH2-p160 heterodimer that restores DNA mismatch repair to tumor cells.* Science, 1995. **268**(5219): p. 1909-12.
162. Palombo, F., et al., *GTBP, a 160-kilodalton protein essential for mismatch-binding activity in human cells.* Science, 1995. **268**(5219): p. 1912-4.
163. Gorbalenya, A.E. and E.V. Koonin, *Superfamily of UvrA-related NTP-binding proteins. Implications for rational classification of recombination/repair systems.* J Mol Biol, 1990. **213**(4): p. 583-91.
164. Walker, J.E., et al., *Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold.* Embo J, 1982. **1**(8): p. 945-51.
165. Dutta, R. and M. Inouye, *GHKL, an emergent ATPase/kinase superfamily.* Trends Biochem Sci, 2000. **25**(1): p. 24-8.
166. Vogelstein, B. and K.W. Kinzler, *Cancer genes and the pathways they control.* Nat Med, 2004. **10**(8): p. 789-99.
167. Kolodner, R.D., C.D. Putnam, and K. Myung, *Maintenance of genome stability in Saccharomyces cerevisiae.* Science, 2002. **297**(5581): p. 552-7.
168. Barzel, A. and M. Kupiec, *Finding a match: how do homologous sequences get together for recombination?* Nat Rev Genet, 2008. **9**(1): p. 27-37.
169. Harfe, B.D. and S. Jinks-Robertson, *DNA mismatch repair and genetic instability.* Annu Rev Genet, 2000. **34**: p. 359-399.
170. Harfe, B.D. and S. Jinks-Robertson, *Mismatch repair proteins and mitotic genome stability.* Mutat Res, 2000. **451**(1-2): p. 151-67.
171. Datta, A., et al., *Dual roles for DNA sequence identity and the mismatch repair system in the regulation of mitotic crossing-over in yeast.* Proc Natl Acad Sci U S A, 1997. **94**(18): p. 9757-62.
172. Saparbaev, M., L. Prakash, and S. Prakash, *Requirement of mismatch repair genes MSH2 and MSH3 in the RAD1-RAD10 pathway of mitotic recombination in Saccharomyces cerevisiae.* Genetics, 1996. **142**(3): p. 727-36.
173. Sugawara, N., et al., *Role of Saccharomyces cerevisiae Msh2 and Msh3 repair proteins in double-strand break-induced recombination.* Proc Natl Acad Sci U S A, 1997. **94**(17): p. 9214-9.
174. Mason, P.J., D.B. Wilson, and M. Bessler, *Dyskeratosis congenita -- a disease of dysfunctional telomere maintenance.* Curr Mol Med, 2005. **5**(2): p. 159-70.
175. Duckett, D.R., et al., *hMutSalpha- and hMutLalpha-dependent phosphorylation of p53 in response to DNA methylator damage.* Proc Natl Acad Sci U S A, 1999. **96**(22): p. 12384-8.
176. Greenwood, M.J. and P.M. Lansdorp, *Telomeres, telomerase, and hematopoietic stem cell biology.* Arch Med Res, 2003. **34**(6): p. 489-95.
177. Maciejewski, J.P. and A. Risitano, *Hematopoietic stem cells in aplastic anemia.* Arch Med Res, 2003. **34**(6): p. 520-7.
178. Brown, K.D., et al., *The mismatch repair system is required for S-phase checkpoint activation.* Nat Genet, 2003. **33**(1): p. 80-4.
179. Hemann, M.T., et al., *The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability.* Cell, 2001. **107**(1): p. 67-77.
180. Teixeira, M.T., et al., *Telomere length homeostasis is achieved via a switch between telomerase- extendible and -nonextendible states.* Cell, 2004. **117**(3): p. 323-35.
181. Bickenbach, J.R., *Identification and behavior of label-retaining cells in oral mucosa and skin.* J Dent Res, 1981. **60 Spec No C**: p. 1611-20.
182. Mills, A.A., et al., *p63 is a p53 homologue required for limb and epidermal morphogenesis.* Nature, 1999. **398**(6729): p. 708-13.
183. Yang, A., et al., *p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development.* Nature, 1999. **398**(6729): p. 714-8.

184. Barrandon, Y. and H. Green, *Three clonal types of keratinocyte with different capacities for multiplication.* Proc Natl Acad Sci U S A, 1987. **84**(8): p. 2302-6.
185. Barrandon, Y. and H. Green, *Cell size as a determinant of the clone-forming ability of human keratinocytes.* Proc Natl Acad Sci U S A, 1985. **82**(16): p. 5390-4.
186. Kucherlapati, R. and R.A. DePinho, *Cancer. Telomerase meets its mismatch.* Nature, 2001. **411**(6838): p. 647-8.
187. Baker, S.M., et al., *Male mice defective in the DNA mismatch repair gene PMS2 exhibit abnormal chromosome synapsis in meiosis.* Cell, 1995. **82**(2): p. 309-19.
188. Blasco, M.A. and W.C. Hahn, *Evolving views of telomerase and cancer.* Trends Cell Biol, 2003. **13**(6): p. 289-94.
189. McEachern, M.J. and E.H. Blackburn, *Cap-prevented recombination between terminal telomeric repeat arrays (telomere CPR) maintains telomeres in Kluyveromyces lactis lacking telomerase.* Genes Dev, 1996. **10**(14): p. 1822-34.
190. Nakamura, T.M., J.P. Cooper, and T.R. Cech, *Two modes of survival of fission yeast without telomerase.* Science, 1998. **282**(5388): p. 493-6.
191. Benetti, R., M. Garcia-Cao, and M.A. Blasco, *Telomere length regulates the epigenetic status of mammalian telomeres and subtelomeres.* Nat Genet, 2007. **39**(2): p. 243-50.
192. Modesti, M. and R. Kanaar, *DNA repair: spot(light)s on chromatin.* Curr Biol, 2001. **11**(6): p. R229-32.
193. Prolla, T.A., et al., *Tumour susceptibility and spontaneous mutation in mice deficient in Mlh1, Pms1 and Pms2 DNA mismatch repair.* Nat Genet, 1998. **18**(3): p. 276-9.
194. Gong, J.G., et al., *The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage.* Nature, 1999. **399**(6738): p. 806-9.
195. Chen, J. and I. Sadowski, *Identification of the mismatch repair genes PMS2 and MLH1 as p53 target genes by using serial analysis of binding elements.* Proc Natl Acad Sci U S A, 2005. **102**(13): p. 4813-8.
196. Wright, W.E. and J.W. Shay, *The two-stage mechanism controlling cellular senescence and immortalization.* Exp Gerontol, 1992. **27**(4): p. 383-9.
197. Lechel, A., et al., *The cellular level of telomere dysfunction determines induction of senescence or apoptosis in vivo.* EMBO Rep, 2005. **6**(3): p. 275-81.
198. Brown, J.P., W. Wei, and J.M. Sedivy, *Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts.* Science, 1997. **277**(5327): p. 831-4.
199. Herbig, U., et al., *Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a).* Mol Cell, 2004. **14**(4): p. 501-13.
200. Artandi, S.E., et al., *Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice.* Nature, 2000. **406**(6796): p. 641-5.
201. Schaetzlein, S., et al., *Exonuclease-1 deletion impairs DNA damage signaling and prolongs lifespan of telomere-dysfunctional mice.* Cell, 2007. **130**(5): p. 863-77.